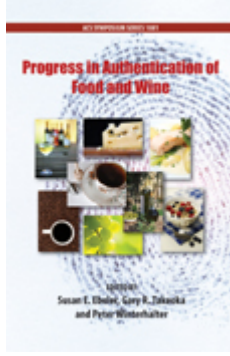
Progress in Authentication of Food and Wine
Susan E. Ebeler, Gary R. Takeoka and Peter Winterhalter

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In Progress in Authentication of Food and Wine; Ebeler, S., et al.;
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Progress in Authentication of Food and Wine

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Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from the ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previous published papers are not accepted.

ACS Books Department

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Preface

In today's global economy there is increasing need for analytical tools to ensure the safety and integrity of our food supply. In the past five years, since the publication of the first ACS proceedings on Authentication of Food and Wine (Ebeler, S. E., Takeoka, G. R., Winterhalter, P., Eds.; *Authentication of Food and Wine*; ACS Symposium Series 952; American Chemical Society, Washington DC, 2007), many issues of food adulteration (*e.g.,* detecting the addition of melamine to foods) and authentication (*e.g.,* confirming region of origin for wines), have reached the forefront of public and regulatory attention. This book compiles the latest research on food and beverage authentication, highlighting the analytical techniques that are being applied to a variety of complex food matrices. The chapters are written by international experts, who participated in an American Chemical Society (ACS) Symposium on "Progress in Authentication of Food and Wine" in San Francisco, CA in March 2010.

After providing a brief overview in Chapter 1 of research progress in the area of food and wine authentication that has occurred over the past five years, Chapters 2, 3, and4 provide a general overview of several techniques that have been widely applied in food authentication studies. These include NMR-based profiling approaches and use of the natural radiocarbon signature for dating foods and beverages and confirming their origin (*i.e*., petrochemical *vs.* biological origin). Application of multivariate statistical tools to analyze the large data sets that are required for authentication of foods and beverages is also discussed.

Authentication of wine and alcoholic beverages is the focus of Chapters 5, 6, 7, 8, 9, 10, and 11. Here, analytical tools such as Fourier Transform Ion Cyclotron Resonance-Mass Spectrometry (FTICR-MS), bomb-pulse ^{14}C signatures, chemical profiles, and sensory analysis are being used to confirm the age, region of origin, and varietal authenticity of wines.

Many food products such as oils, vinegars, cheeses, and caffeinated beverages are labeled and sold, based not only on the plant or animal source, but also based on protected designations for region of origin and processing methods; however analytical tools to monitor these claims have been limited. There is also increasing need for analytical methods to monitor foods enriched with various phytonutrients such as sterols and stanols and to detect fraudulent adulteration with components such as melamine. Chapters 12, 13, 14, 15, 16, and 17 focus on approaches for authentication of these ingredients and food matrices.

Approaches for determining quality and authenticity of fruit juices and fruit products are the focus of Chapters 18, 19, 20, and 21. Here, analytical methods, such as infrared spectroscopic techniques, NMR profiling, and chemical profiling of volatiles, sugars and other carbohydrates, polyphenols, etc. are allowing for the rapid and sensitive

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determination of the overall fruit content and composition in juices, concentrates, and fruit preparations such as jams, jellies, and spreads.

Finally, methods to confirm authenticity and safety of spices (*e.g.*, saffron) and botanicals are the focus of Chapters 22, 23, and 24. Analytical challenges associated with detecting chemical adulteration of spices, screening complex matrices for pesticide residues, and applying DNA-based approaches for species and varietal authenticity of botanicals are discussed.

While regulatory agencies are demanding improved methods to ensure that foods comply with labeling and safety requirements, consumers also desire more knowledge of where foods are produced and how they are processed. The chapters in this proceedings highlight the many analytical approaches and the complex challenges that researchers are facing as they strive to ensure the safety and integrity of our food supply in order to meet these regulatory needs and consumer demands. As outlined in these chapters, chemical, sensory, molecular biology, and advanced statistical tools are all being employed to characterize food composition. Symposia such as the one on which this proceedings are based, are proving invaluable for bringing together scientists from many different disciplinary areas to share ideas and identify new approaches and techniques that can be used to address these complex scientific challenges.

Since the first symposium on "Authentication of Food and Wine" in 2005 (Ebeler, S. E., Takeoka, G. R., Winterhalter, P., Eds.; *Authentication of Food and Wine*; ACS Symposium Series 952; American Chemical Society, Washington DC, 2007), much progress has been made. However, unfortunately, opportunities for fraud and adulteration remain, and innovative analytical methods will always be needed to ensure the safety and integrity of our food. We hope that this volume will prove a valuable resource for researchers, regulators and industry scientists who are at the forefront of responding to authenticity and adulteration claims in our food supply.

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Chapter 1

Progress in Authentication of Food and Wine

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Authentication of foods, wines and beverages for varietal, country- (or region-) of-origin, and processing conditions is becoming of increasing concern to consumers and regulators both in the U.S. and internationally. As markets become more globalized and foods and beverages are sourced from many locations outside of the U.S., the need for information on product authenticity is becoming mandated either by legal regulations or by market demand. However, there are significant chemical and analytical challenges faced in confirming food authenticity and determining food adulteration. Here we highlight some recent cases that emphasize the need for improved analytical information that will limit economic fraud and improve food safety.

Introduction

As reviewed by Winterhalter (*1*), adulteration of foods and beverages has been a problem since the beginning of food commerce. As a result, regulations have developed to ensure the safety and integrity of our food supply and there have been concomitant improvements in analytical tools to ensure that food composition is consistent with labeling and consumer expectations.

The authenticity of numerous products has been studied (Table I). Details of the authenticity testing for various foods and wines are discussed in the following book chapters. Some methods that have been used for authenticity testing are listed in Table II and have also been discussed in a recent book (*2*).

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Table I. Examples of products that have been investigated for authentication

Edible oils, olive oil, hazelnut oil, wine, tequila, coffee, tea, milk, cheese, milk powder, fruit juices, lemon juices and concentrates, apple juices, blood orange juices, (E)-α(β)-ionone from raspberries, α-ionone, β-ionone from various sources, bitter almond oil, cinnamon oil, natural vanilla flavorings, ginseng, *Echinacea* root, balsamic vinegar, rice, honey, hazelnuts, fish, anchovy, tuna, sole, sardine, salmon, razor clams, fish oils capsules, mustard oils

Table II. Examples of methods used for authentication

HPLC, GC-MS, electronic nose, MALDI-TOF MS, electrospray ionization (ESI) FT ion cyclotron resonance MS (FT-ICR MS), capillary gas chromatography-isotope ratio mass spectrometry in the combustion and pyrolysis modes (HRGC-C/P-IRMS), stir bar sorption extraction enantio-multidimensional gas chromatography mass spectrometry (SBSE enantio-MDGC-MS), UV-Vis, FT-Raman, FT-MIR, FT-NIR, front-face fluorescence spectroscopy, site-specific natural isotope fractionation-nuclear magnetic resonance (SNIF-NMR), ^{1}H NMR, polymerase chain reaction-enzyme linked immunosorbent assay (PCR-ELISA), sequence tagged site-restriction fragment length polymorphism (STS-RFLP), PCR-RFLP, optical thin-film biosensor chips, lab-on-a-chip capillary electrophoresis (CE), cleaved amplified polymorphic sequence (CAPS) based assay, differential scanning calorimetry (DSC), sensory evaluation

For example, a wide variety of methods has been used to determine the authenticity of olive oil. Spectroscopic methods such as infrared (IR) (*3*), near-infrared (NIR) (*4*), mid-infrared (*5*), and Raman (*6*), combined with chemometrics have been employed to detect adulterants in olive oil. Vigli et al. (*7*) used a combination of ^{1}H NMR and ^{31}P NMR spectroscopy and multivariate statistical analysis to classify 192 samples from 13 types of vegetable oils, namely hazelnut, sunflower, corn, soybean, sesame, walnut, rapeseed, almond, palm, groundnut, safflower, coconut, and virgin olive oils. 1,2-Diglycerides, 1,3-diglycerides, the ratio of 1,2-diglycerides to total diglycerides, acidity, iodine value, and fatty acid composition determined on analysis of the respective ^{1}H NMR and ^{31}P NMR spectra were selected as variables to establish a classification/prediction model by employing discriminant analysis. Fresh virgin olive oils extracted from olives of normal ripeness are characterized by high 1,2-diglycerides to total diglycerides ratio ($D \geq 0.90$). Use of fresh virgin olive oil samples ($D \geq 0.90$) allowed detection of adulteration as low as 5% w/w with this NMR method. Electrospray ionization (ESI) Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) has been used to resolve and identify thousands of distinct chemical components of commercial canola, olive and soybean oils without prior chromatographic separation (*8*). In negative-ion ESI FT-ICR MS, the acidic components of soybean oil are easily distinguished from those of canola and olive oil based on relative abundances of C_{18} fatty acids (the most abundance species in canola and olive oil is $C_{18:1}$, while in soybean oil it is $C_{18:2}$), whereas olive oil differs from canola and soybean oil based on relative abundances of tocopherols (β,γ-tocopherol has a high relative abundance in both canola and soybean oil but low relative abundance in olive oil). In positive-ion

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ESI FT-ICR MS, the three oils are readily distinguished according to the relative abundances of di- and triacylglycerols with various numbers of double bonds in the fatty acid chains. This technique does not require chemical pretreatment, extraction or chromatographic separation yet provides a new way to characterize vegetable oils and to detect and identify adulterants. Sánchez-Hernández and co-workers (*9*) developed a capillary electrophoresis method with UV detection for the determination of trigonelline (*N*-methylnicotinic acid) in seeds and vegetable oils. Trigonelline was determined in soy and sunflower seeds and their respective oils but was not detected in olives or olive oils. Trigonelline was proposed as a novel marker for the detection of olive oil adulteration with vegetable oils such as soy and sunflower oils. There has been a growing interest in the application of DNA-based markers since they are independent from environmental conditions. Specific protocols for DNA isolation from olive oil have been developed (*10–13*). qRT-PCR is a useful tool in the development of molecular markers for olive oil authentication and it should be used for the optimization of critical parameters such as the amplicon size and the DNA isolation method (*14*). Plastid based molecular DNA technology has been proposed as a rapid method for the detection of adulterants such as canola and sunflower oils in olive oil (*15*).

Recent Examples

The adulteration of coffee with cereals, coffee twigs, brown sugar, etc. is considered to be a serious problem affecting Brazilian coffee quality with corn being considered as the mostly widely used adulterant. In response to this problem, researchers at the National Center for Agricultural Utilization Research in Peoria, IL developed an HPLC method measuring tocopherols to detect coffee adulteration with corn (*16*). Six coffee varieties were analyzed by HPLC and were found to contain the following percentages of α-, β-, γ-, and δ-tocopherol: 29.0, 61.7, 3.3, and 6.0, respectively. Six roasted corn samples contained the following percentages of α-, γ-, and δ-tocopherol: 3.6, 91.3, and 5.1, respectively. These differences in tocopherol content were applied to detect corn in a pure coffee sample intentionally contaminated with corn with the best result obtained with γ-tocopherol. Using this methodology, the researchers found one coffee sample that was apparently adulterated (8.9%), mostly likely with corn. Though the results were considered preliminary, it appeared that γ-tocopherol fingerprinting has the potential as a marker to detect corn adulteration in coffee.

The adulteration of traditional Basmati (TB) rice varieties with evolved Basmati (EB) varieties and long-grain non-Basmati (NB) varieties is performed by unscrupulous traders to earn additional profits. Basmati rice is a highly prized rice on the international market. Originated in the Himalayas, Basmati is a long grain rice with a pleasant and distinct aroma and soft and fluffy texture when cooked. Unfortunately, Basmati also has undesirable traits such as tall stature, low yield and photosensitivity. Attempts since the 1970s to develop high yielding Basmati rice varieties by cross breeding resulted in many high yielding EB varieties. However, they did not match the quality standards of the TB varieties.

TB (i.e., Basmati370, Dehradum, and Taraori) varieties command a higher price ($850/MT) compared to EB varieties (i.e., Pusa Basmati, Mahi Basmati and Super Basmati) ($480-520/MT) while NB varieties (i.e., PR106) have even a lower value ($106/MT) (*17*). Adulteration is a problem since it is difficult to differentiate between TB, EB and NB rice on the basis of visual observation or physiochemical tests. To solve this problem, Vemireddy and co-workers (*17*) developed a capillary electrophoresis (CE) based microsatellite DNA profiling protocol which facilitates quick and accurate detection and quantification of the adulteration of Basmati rice. The single-tube assay multiplexes eight microsatellite loci viz., RM1, RM55, RM44, RM348, RM241, RM202, RM72 and RM171 to generate variety specific allele profiles that can detect adulteration from 1% upwards.

A recent case of food fraud involved the false labeling of "industrial eggs" (eggs that do not meet the quality requirements of sale to the public but instead can only be used in processed foods once liquefied) and battery eggs (eggs produced by chickens in cages) as free range or organic. Between June 2004 and May 2006, a wholesaler, Keith Owen who ran Heart of England Eggs Unlimited, supplied about 100 million falsely labeled eggs to bigger packing companies which, in turn, provided the majority of eggs to the major supermarkets and numerous small shops. When inspectors checked a selection of Owen's allegedly free range eggs with UV light they found wire marks that showed that the eggs had been laid on a metal cage, not on a bed of straw. Mr. Owen was sentenced to three years in jail and was also ordered to pay £250,000 in costs and to settle a £3 million confiscation order within 12 months.

Wine is commonly sold with labeling information regarding the grape variety used, the appellation or grape growing region, and the vintage or age—and price and consumer expectations are frequently determined based on this information. Two recent, high profile examples highlight the need for improvements in our ability to authenticate the labeling information for any given bottle of wine. In the first instance, concerns have arisen that some high-end, collectible wines sold at auction for thousands of dollars per bottle were actually counterfeit, cheaper wines, bottled to look like their authentic counterparts (*18–20*). On the other end of the price scale, producers in France have been convicted of replacing Pinot noir wine with cheaper Merlot and Syrah wines; approximately 13.5 million liters of the mislabeled Pinot noir wine was sold to Gallo Winery in the U.S. which was then incorporated into millions of bottles of popular Gallo wine brands (*21*, *22*). In these, like most cases, confirmation of the fraudulent claims is difficult however and often relies on following production records—which may no longer exist or may not be complete in the case of many older collectible wines.

Challenges

The above examples point to an increasing need to develop analytical tools and information that can be used to ensure that foods, wines, and beverages that consumers obtain in the marketplace are consistent with label information and while many successful analytical methods have been developed, several challenges remain.

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One of the main challenges for authentication of foods and beverages is the need for accurate reference materials and standards that can be used to ensure proof-of-identity. The difficulties in developing these authentic standards are exemplified in the Chilean wine industry where many vineyard plantings of Merlot grapevines actually turned out to be plantings of Carménère (*23*, *24*). Because of similarities in the visual appearance of these two varieties, the misidentifications were not recognized until the late 1990's when methods were developed for differentiating different grape cultivars using specific DNA markers. Use of DNA markers has now become one of the most reliable means to confirm species identification of plant and animal materials (*25–28*) and has also been used to confirm the existence of allergens and GMOs in the food supply (*29*, *30*). Therefore, when possible, DNA markers should be the first method used to confirm species or cultivar identify before further instrumental analyses are developed and applied to differentiate chemical profiles such as cultivar differences in polyphenol composition in grape. In practice, proof-of-identity using DNA markers is limited for many foods since the DNA can be readily degraded during storage and processing, particularly during thermal processing and/or as a result of enzymatic and acidic degradation reactions (*31*, *32*). However, new extraction methods and fragment sequencing procedures are being developed which may make it possible to obtain DNA fragments from highly processed matrices (e.g., wines), for use in PCR-based authentication procedures (*33*, *34*).

One area where there has been significant progress is the development of certified standard reference materials for the dietary supplements and botanicals industries where Good Manufacturing Practices (GMP) regulations require that manufacturers establish specifications for identity and purity of the supplement and set limits on contaminants and adulterants (*35*). In the U.S., the National Institute of Standards, in conjunction with the NIH Office of Dietary Supplements, and the FDA Center for Drug Evaluation and Research is developing Standard Reference Materials (SRMs) for dietary supplements. These SRMs must meet proof of identity criteria (such as confirmation by DNA markers), have well defined sample characteristics (i.e., ground, dried, sieved to a certain size, etc.), be packaged to ensure chemical and microbial stability, and be analyzed to ensure that the matrix and material represents certified values for active ingredients (*36*). Development of these and similar commercially available certified materials will be a valuable tool to improve the analytical chemist's ability to identify unknown botanical products in the future.

Along with reference materials, authentic chemical reference standards are needed for accurate quantitation and characterization of food composition. For example, phenolic glycosides and chiral compounds are often used for authentication of foods and essential oils (reviewed in (*37*)), however, standard chemical reference compounds of known structure and chirality are often unavailable or of insufficient purity to confirm their identification in unknown samples. Del Mar Caja et al. (*38*) have further demonstrated that 'natural' and 'synthetic' sources of many chemicals differ in their ™ $^{13}C_{V\text{-}PDB}$ and ™ $^{2}H_{V\text{-}SMOW}$ values therefore accurate sourcing information for many chemicals may be needed if isotope ratio analysis is used for authentication of foods and

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essential oil components. When the standards are not readily available or not-well characterized, additional analyses or further purification are needed to determine the identity of compounds in unknown samples, adding significantly to analysis cost and time.

Further challenges arise from the natural variation in the chemical composition of most foods and beverages. Because this natural variation can be quite extensive, procedures for authentication of foods and beverages will require creation of large databases of analytical information that represent the range of matrices, analyte concentrations, and processing or storage conditions that might be expected for a given sample type (e.g., to authenticate wines of a given grape variety, wines with a range of residual sugar and alcohol levels should be included in the analytical databases). Creation of these databases will require large numbers of samples and, in most cases, information on multiple analytes (e.g., both volatile and non-volatile analytes). Using powerful statistical tools, the compositional information in the databases can then be used to develop models that will allow unknown samples to be differentiated based on the criteria of interest. For example, although PCA has been widely used as a multivariate statistical tool for authentication studies, Cuny et al. (*39*) used the recently developed Independent Component Analysis (ICA) technique to process complex data from ^{1}H NMR spectroscopy and showed that ICA was better able to discriminate between orange juice and grapefruit juice samples (and their mixtures) than PCA. In another example, artificial neural networks were successfully used to verify the geographic origin of commercial mineral waters based on the chemical ion composition (*40*). Finally, two chemometric approaches, principal component analysis (PCA) and linear discriminant analysis (LDA), successfully classified different South African wines based on the grape cultivar using combined data from the volatile composition and mid-infrared spectral profiles (*41*). In summary, while much progress has been made in developments and applications of databases and multivariate statistical tools, most databases to date are still limited to relatively small numbers of related samples or to a small number of analyte profiles. In addition, because blending is allowed for many foods and beverages (e.g., up to 15% of a wine can be of a variety other than that indicated on the label; www.ttb.gov), more work is needed to develop databases and corresponding statistical models that can identify and distinguish these blends.

Finally, in order to obtain the vast compositional information from such large numbers of samples, improved analytical tools that rapidly, accurately, and sensitively profile large numbers of analytes are of increasing interest and are finding wide application for food authentication. Some promising recent analytical approaches have utilized Laboratory-on-a-Chip Capillary Electrophoresis for DNA-based assays (*42*); multi-chemical sensor arrays including "electronic noses" and "electronic tongues" (*43*, *44*); and rapid MS-based profiling procedures (*8*, *45*, *46*) to authenticate a range of foods and beverages. Increasingly analytical instrumentation manufacturers are recognizing the need for development of new high-throughput tools for detecting food adulteration and for addressing authentication issues and we can expect to continue to see advances in this area (*47–49*).

In summary, while there have been many advances in analytical tools for determining the authenticity of a food or beverage, cases of economic fraud and adulteration still occur and will continue to challenge the analytical chemist. By highlighting the current state-of-the-art as well as emphasizing the need for additional research in this area scientists will play an important role to ensure that our food supply is safe and that consumers will be truly getting what they pay for.

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Chapter 2

NMR Studies in Food Authentication

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Different food matrices were analyzed by means of NMR spectroscopy and chemometrics, with the aim of authenticity determination. Some foods were investigated in terms of geographical discrimination, while others in fraud detection. In all cases studied, the metabolic water soluble content of the analyzed foods, was investigated by means of ^{1}H and ^{13}C spectroscopy, highlighting the powerful capacity of this "omic" approach in food authenticity determination. Our results suggested in case of geographical determination that foods carry a sort of "memory" of the soil were they were grown, which is transferred into the metabolic content, thus enabling the detection and the discrimination.

Introduction

In the last years several studies have been performed about food characterization, frauds and adulteration detection; in other words the so-called "food authentication" process. An increasing interest from both consumers and producers concerning particularly high quality and trusted geographical origin food products within Europe and all around the world has undoubtedly grown in the last years. The reasons for this can be traced to patriotism, health benefits or specific organoleptic and culinary qualities associated with regional products, media attention, decreasing confidence in quality and safety products coming from outside their local regions, countries or EU and the concern about animal welfare and environmentally friendly production methods (*1*).

EU plays a primary role in enhancing high quality attributes and in sustaining this wide range of cultures and culinary traditions by introducing regulations

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and systems to promote and to protect geographical indications and designations of origin for agricultural products and foodstuffs. These regulations allowed the application of different quality indications to food products and precisely "Protected Designation of Origin" (PDO), "Protected Geographical Indication" (PGI) and "Traditional Speciality Guaranteed" (TSG) (*2*).

The products that have achieved these trademarks have therefore quality levels that could: a) enhance food security, inasmuch as they contribute to rural development and support small producers for accessing markets, b) complying with food safety requirements, assuring added-value attached to the product's specifications, c) contribute to social welfare and food diversity and biodiversity preservation. Indeed, thanks to the link between such products and their areas of origin, they can help to preserve local resources, maintain traditions, strengthen the organization of local stakeholders and to prevent delocalization and rural exodus.

Food characterization is becoming very challenging because it includes both authenticity and geographical origin determinations. Among all possible approaches, metabolomic has shown its large potentiality in this respect. As a matter of fact, the capability of metabolic profiling has been revealed to be one of the most informative tools in determining the composition, adulteration and quality of foods simultaneously. The metabolite content constitutes a sort of a "fingerprint" for each single food product, representing a useful tool also in geographical food characterization.

NMR techniques have already been demonstrated to be well suited for analyzing all metabolic content of a food matrix: with a single experiment all soluble chemical compounds can be identified and characterized in a very short time. In the present work we present some studies performed by combining NMR techniques and multivariate statistical analysis protocols on different food matrices in order to guarantee their authentication in terms of quality and geographical origin determination.

Results and Discussion

1. Parmigiano Reggiano

Parmigiano Reggiano cheese, which got the PDO trademark in 1996, is certainly one of the most appreciated Italian food all over the world. During ripening cheese components (proteins, lipids and sugars) undergo important chemical, physical and enzymatic modifications leading to a particular and highly valuated food product. In our study we analyzed the water soluble content of commercially available Italian Parmigiano Reggiano cheeses at different ripening stages (14, 24 and 30 months) and "Grana type" cheeses samples coming from Eastern Europe, the more prevalent type on the Italian market. The typical ^{1}H NMR spectrum of Parmigiano Reggiano cheese with the principal spin system assignments is reported in Figure 1.

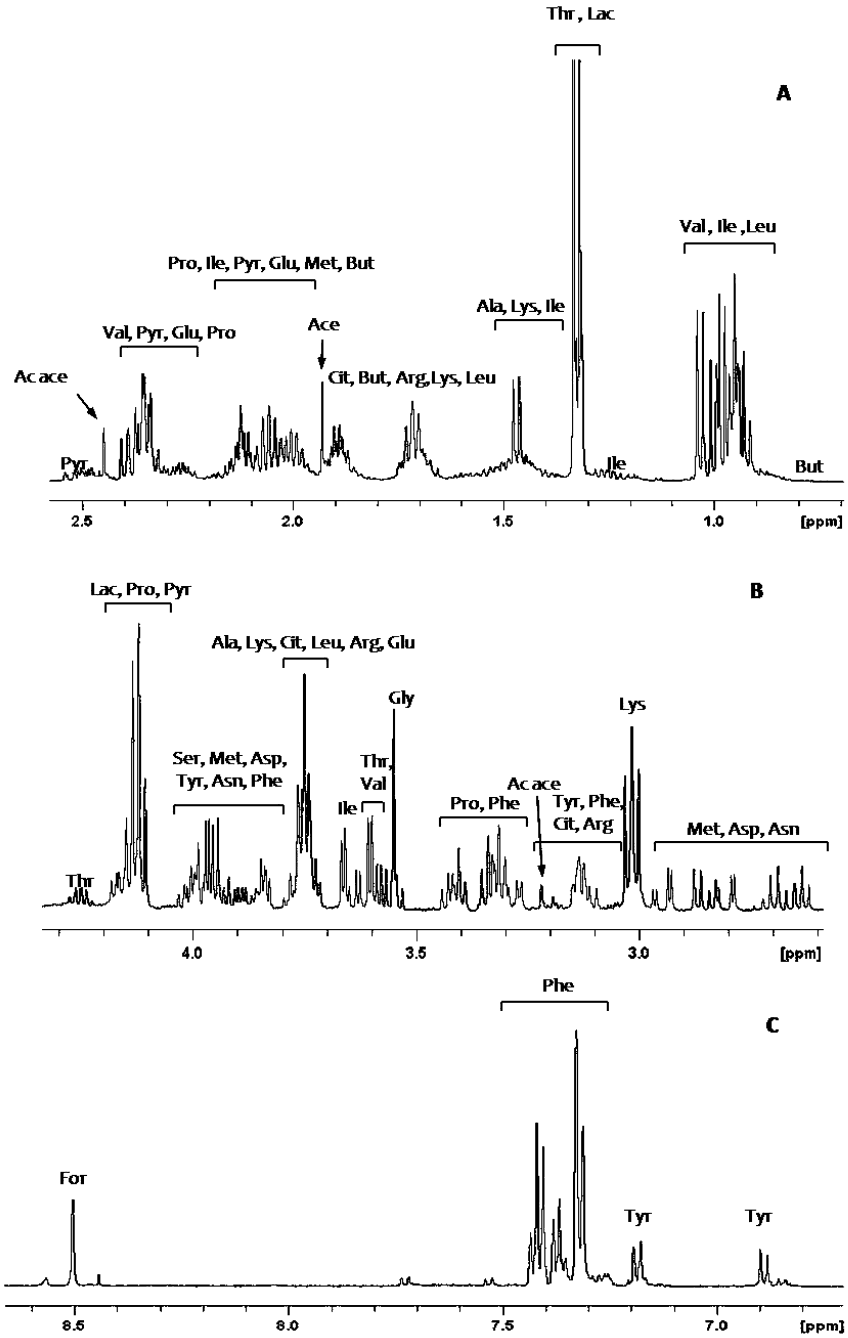

Figure 1. [1]H NMR spectrum of "Parmigiano Reggiano" aqueous extract sample. Aliphatic (1A and 1B) and aromatic (1C) region with principal spin system assignments are reported.

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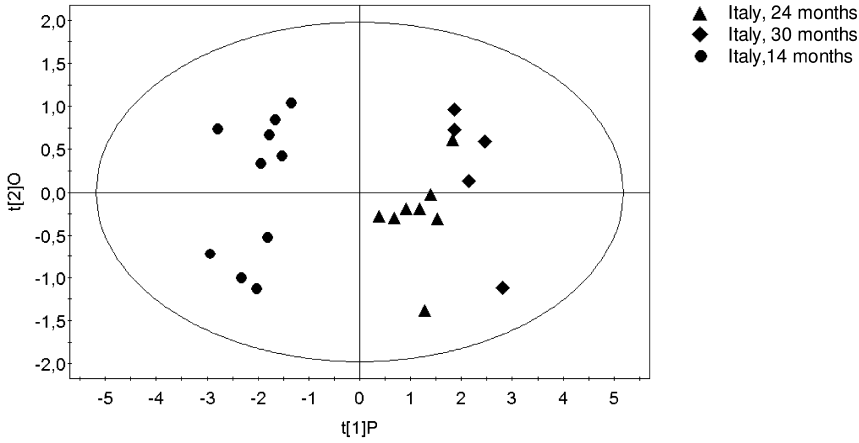

Figure 2. O-PLS score plot performed by considering all Italian "Parmigiano Reggiano" cheese samples of 14 months (dot), 24 months (triangle) and 30 months (diamond).

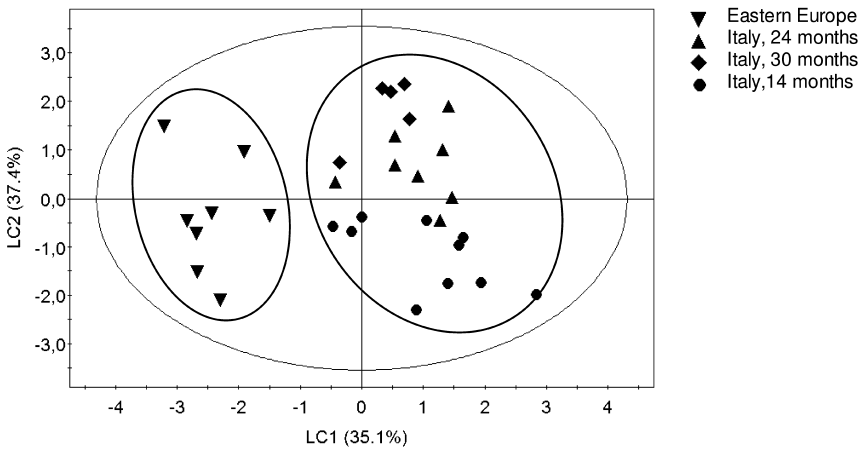

Figure 3. PLS-DA score plot performed by considering all Italian "Parmigiano Reggiano" cheese samples of 14, 24 and 30 months (dot, trangle and diamond respectively) and foreign "Grana type" cheese samples (inverted triangle).

Initially, only Italian cheese samples were considered and NMR data evaluated with O-PLS. A very clear differentiation among samples according to different ripening stages was feasible with two components accounting for 71.8% of the total variance and Q^2=89.3% (score plot in Figure 2). The score contribution plots, for the analyzed samples, revealed an increase in amino acids content with ripening process: in particular younger samples showed the highest content in leucine and isoleucine; samples aged for 30 months were enriched in threonine compared to samples aged for 24 months which conversely contained a higher amount of isoleucine. Comparing samples aged for 24 and 14 months,

higher amino acid content was generally observed for older cheeses while younger ones had higher levels of leucine.

Furthermore, PLS-DA was performed by considering all Italian and foreign cheese samples obtaining a clear cut differentiation according to the geographical origin with the 72.5% of the total variance explained by the first two latent components (score plot in Figure 3). The corresponding loading plot highlighted leucine, isoleucine and also lactic acid, butanoate and acetic acid as the characteristic compounds for foreign samples. Conversely, Italian samples were characterized by higher amount of all other compounds, particularly threonine (which typified 30 months Parmigiano Reggiano samples), valine, proline, glutammic acid, lysine, alanine, serine, arginine and citrulline (*3*).

2. Honey

Honey is a very popular and appreciated product all around the world because of its readily available source of energy, and also because of its antibacterial and antioxidant activity (*4*, *5*). It is mainly constists of a complex sugar mixture of variable composition based on different brands; minor components are amino acids and aromatic compounds with different composition among the varieties. At present, the botanical and the geographical origin of honey are determined by melisso-palynological analysis even if some limitations are present: in fact, great knowledge of pollen morphology and specialised professional personnel are required to reliable results (*6*). Honey is a food product often subjected to adulteration (mainly due to the lower price of honey from China or South America) and thus quality and authenticity controls are necessary to preserve the production zone in developing particular standards of quality and for protection of consumers from commercial speculation (*7*). Differences in price and quality are present between countries within Europe and even among regions in the same country. For controlling and verifying the quality specifications for different honeys and for different geographical origins, new and objective analytical methods are required. The results of our study concerning greographical origin determination by using ^{1}H NMR spectroscopy combined with chemometrics, are reported below. This approach allowed us to detect sugars, amino acids and organic acids (Figure 4). Polyfloral, high mountain polyfloral, rhododendron and acacia honey samples coming from different countries or from different Italian regions were considered. Polyfloral honey samples coming from different countries were clearly discriminated by performing PLS-DA analysis (score plot in Figure 5). Successively, a hierarchical PLS-DA model was created for Italian, Argentinian and Hungarian polyfloral honeys of certain origin constituting a training set. A good classification of honey samples according their geographical origin was then achieved. Test set samples including Argentinian, Italian and Hungarian honey, blend of honeys from Euroropean countries and blend of honey from European and non European countries were reprojected onto the hierarchical PLS-DA model to check whether they could be classified with respect to training set samples. The 3D score plot of the predicted *t* scores (tPS) in Figure 6 shows the good prediction for test set samples (open symbols) from Italy, Argentina and Hungary. Noticeably samples of mixed origin (EC and non-EC countries) were

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clustered in the middle of the score plot confirming their mixed content. Blended samples from EC countries resulted in the Hungary grouping most likely due to its nonbalanced mixed composition, enriched in Hungarian honey.

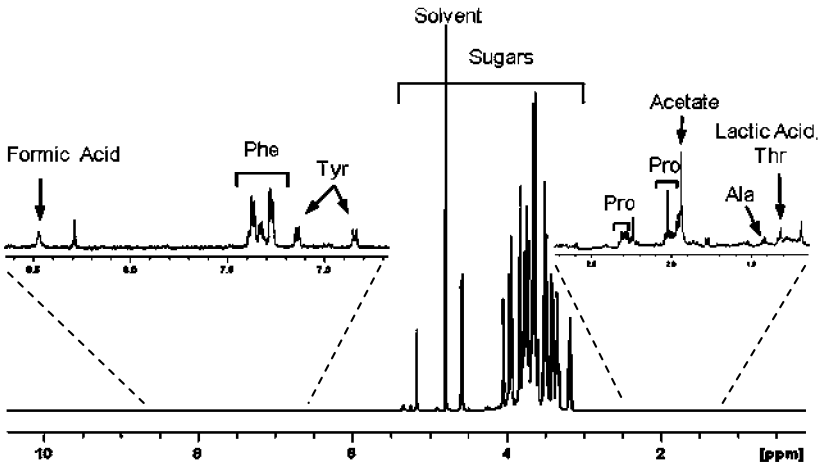

Figure 4. [1]H NMR spectra of honey sample with main assignments reported.

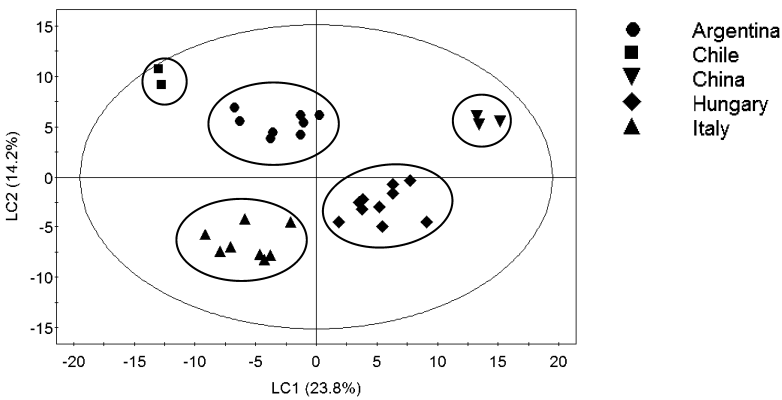

Figure 5. PLS-DA score plot performend by considering polyfloral honey samples from Argentina (dot), Chile (box), China (inverted triangle), Hungary (diamond) and Italy (triangle).

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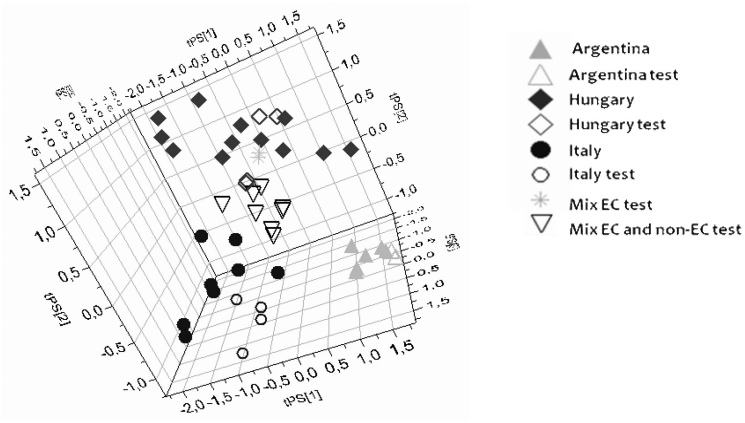

Figure 6. 3D Hierarchical PLS-DA score plot performed considering polyfloral honey of certain origin (training set) with re-projection of polyfloral test set samples scores. Filled symbols represent training set honey samples from Hungary (diamond), Italy (circle) and Argentina (triangle) while open symbols represent test set honey samples from Hungary (diamond), Italy (circle), Argentina (triangle), from different EC countries (star) and from different EC and non-EC countries (inverted triangle).

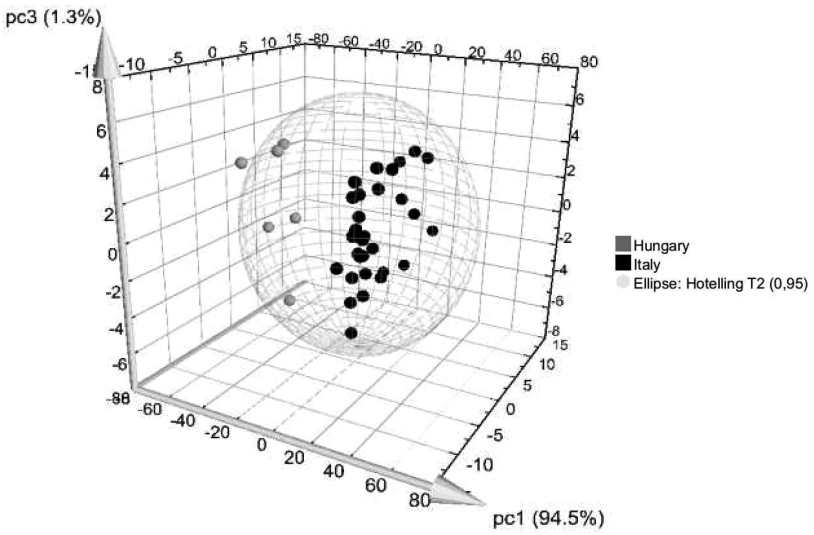

Figure 7. 3D PCA score plot performed by considering acacia honey samples coming from Italy (black) and Hungary (light grey).

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Preliminary studies were also performed also by considering polyfloral and rhododendron honey producted over 1000 meters of altitudine in different regions of northern Italy. PLS-DA performed on NMR data led to a very good differentiation among polyfloral honey samples from Lombardia, Piemonte and Trentino Alto Adige regions. Rhododendron honey samples coming from Lombardia, Piemonte and Valle d'Aosta regions were discriminated as well (data not shown). Finally, Acacia honey samples coming from Italy and Hungary were analyzed. The PCA results were useful in discriminating samples according to their origin as shown in the score plot of Figure 7. The corresponding loading plot indicated a generally higher content of all water-soluble compounds for Italian samples with respect to foreign ones (*8*).

3. Concentrated Tomato Paste

Concentrated tomato paste is obtained from tomato fruits which after harvesting, washing, treating with cold or hot break process and after seed and peel removing, are subjected to concentration with heat exchangers until at least 12, 18, 28 and 36° Brix are obtained for semi, mono, double and triple concentrated tomato paste respectively. Interestingly large amounts of triple concentrated tomato paste are imported in Italy from China, one of the world's largest tomato producers. However, very few indications support the possibility of identifying the origin and the quality of different tomato products (*9*, *10*).

Actually, Italian law (*11*) only requires tomato sauces producers to indicate the place of origin of tomato fruits on the label. In this contest, many frauds about the real origin of tomato products were made and in this respect a growing interest from both consumers and producers about food geographical characterization increasing at the present (*1*, *12*).

We analyzed by ^{1}H NMR (an expansion of the aliphatic region of the typical ^{1}H NMR spectrum of water extract of triple concentrated tomato paste sample with assignment is reported in Figure 8) more than 100 double and triple concentrate tomato paste samples coming from Italy and China producted in both 2007 and 2008.

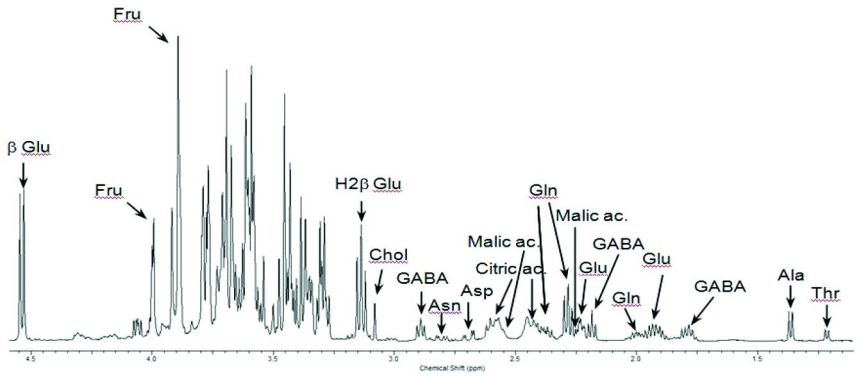

Figure 8. ^{1}H NMR spectrum of triple concentrate tomato paste aqueous extract sample. Aliphatic region with principal spin system assignments are reported.

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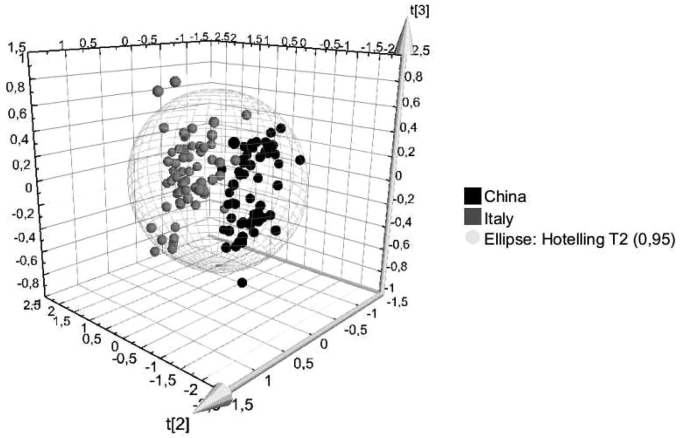

Figure 9. 3D PCA score plot performed by considering 119 double and triple concentrate tomato paste samples produced in 2007 and 2008 coming from Italy (grey) and from China (black).

Notwithstanding different tomato cultivars and ripening stages were usually used to obtain the final product, as well as different concentration rate (double and triple) and different years of production were considered in our analysis, an excellent discrimination of samples based on their geographical origin was achieved by performing the unsupervised PCA analysis on the water soluble metabolite content of lyophilized samples (Figure 9). To build a robust classification model with robust predictivity, samples were also divided into balanced training and test sets using D-Optimal Onion Design. O2PLS-DA protocol was performed on the training set samples. A correct classification of both training and test set samples according to their geographical origin was achieved independently from both year of production and concentration rate. On the basis of the resulting S-plot, Italian concentrated tomato paste samples were characterized by GABA (γ-aminobutyric acid), glucose and alanine while Chinese samples were characterized by fructose, glutamine and glutamic acid (data not shown) (*13*, *14*).

4. Extra Virgin Olive Oil

Olive oil represents a fundamental component of the Mediterranean diet. It is well appreciated for its organoleptic characteristics and its large content of antioxidant compounds. Different quality products are present in the market because of different methods for extracting the oil from olives, pedoclimatic conditions, agronomic factors, cultivar and processing techniques. Extra virgin olive oil (EVOO) in particular presents superior organoleptic characteristics and nutritional properties that make it an highly valuable food. For this reason, several types of fraud based on addition with less expensive oils are encountered. To protect and to valorize their EVOO products some producers obtained the PDO trademark. PDO status refers to an agricultural product or foodstuff which

is produced, processed and prepared in a given specific geographical area, by using recognized know-how and whose qualities or properties are significantly or exclusively determined by the geographical environment, including natural and human factors.

In our study we analyzed different EVOOs coming from the two banks of Lake Garda (the Brescia and the Verona bank) either belonging to Garda PDO and EVOOs coming from the Lake Iseo in the Brescia province. The recorded ^{1}H NMR extra vergin olive oil spectrum was dominated by fatty acids signals; minor components, like aldehydes and terpenes, are revealed in appropriate ratio when very accurate recording conditions are satisfied. Performing hierarchical PLS protocol on selected resonance and precisely two terpenes, cyclo-artenole, hexenal and trans 2-hexenal, a clear discrimination between EVOO samples from Lake Garda and Lake Iseo was achieved. Interestingly, a good differentiation was obtained between EVOO samples coming from the two banks of Lake Garda though they were grown in close proximity but were produced in different soils. These differences affected the metabolic content of EVOO samples constituting a fingerprint for the geographical differentiation of this valued food product (score plot in Figure 10) (*15*).

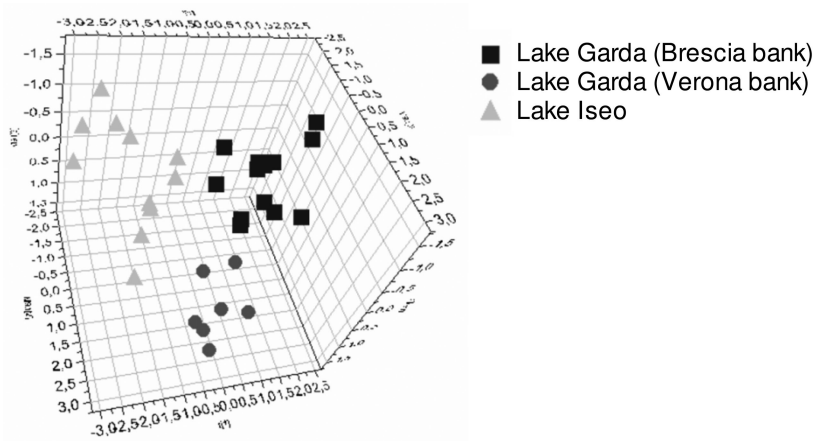

Figure 10. 3D hierarchical PLS score plot performed by considering extra virgin olive oil from PDO Lake Garda (box for Brescia bank and dot for Verona bank) and from Lake Iseo (triangle).

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5. Balsamic and Traditional Balsamic Vinegar of Modena

Balsamic and Traditional Balsamic vinegars of Modena (BVM and TBVM respectively) are two well known and appreciated Italian food products all over the world. BVM which obtained the PGI trademark in 2009 (Reg. CE n° 583/2009 of 3 July 2009) is made from wine vinegar with the addition of caramel and a small quantity of aged wine vinegar, as described in the set rules (D.M., December 3, 1965) for the preparation procedures. BVM samples can then experience a further ageing process (usually they are classified as "refined" or "aged" according to the experienced < 3 or > 3 years of ageing process), thus reaching higher quality parameters, but always deeply different from TBVM quality.

TBVM obtained the PDO trademark in 2000 (Reg. CE n° 812/2000, GUCE L. 100 of 20 April 2000) and is a completely different product. It is made only of cooked must which is left to age in wooden barrels, of decreasing size and different wood type, for at least 12 years before sale but it can reach more than 25 years, giving rise to the "extra old" product. During this ageing process the cooked must experiences several chemical modifications such as sugar degradation, acetylated derivatives formation, enrichment of aroma from barrels etc. For both BVM and TBVM, several different frauds, essentially set rules violations, have been encountered such as falsification of ageing process, sugar and thickener. Analytical controls are nowadays obtained by means of sensorial analysis and very simple chemical–physical property determinations, like total acidity, density and dry residual that, in our opinion, cannot fully address the fraud and the authentication problem. To evaluate the possibility of a deeper BVM and TBVM characterization in terms of quality, ageing and also in terms of fraud detection, we performed several studies by combining NMR and multivariate statistical protocols. A typical ^{1}H NMR spectrum of BVM is reported in Figure 11 with the expansions of aliphatic and aromatic regions. The relaxation analysis of BVM samples obtained by measuring the spin-lattice relaxation time (T1) of acetic acid and β-glucose (*16*) led to a PLS model based on these measurements combined with quantitative determination of five selected metabolites, namely ethanol, acetic acid, malic acid, glucose and HMF. The model resulted in very good BVM ageing process determination differentiating >3 and < 3 years BVM samples (data not shown).

Furthermore, seventy-two BVM and TBVM samples with different ageing process were analyzed (*17*). The hierarchical PLS-DA model performed on NMR data, resulted in a high predictable capability statistical model in terms of ageing, demonstrating a very good discrimination among BVM < 12 years, TBVM > 12 and > 25 years and TBVM < 25 years of ageing. The validation model was checked on both training and test sets and further confirmed by accurate prediction of 41 unknown samples (data not shown).

The corresponding loading plot suggested acetate, ethanol and 3-hydroxy-2-butanone as the variables positively affecting "young" samples (< 12 years) while sugars and HMF are the variable characterizing the "not young" samples (>12 and <25 years and >25 years).

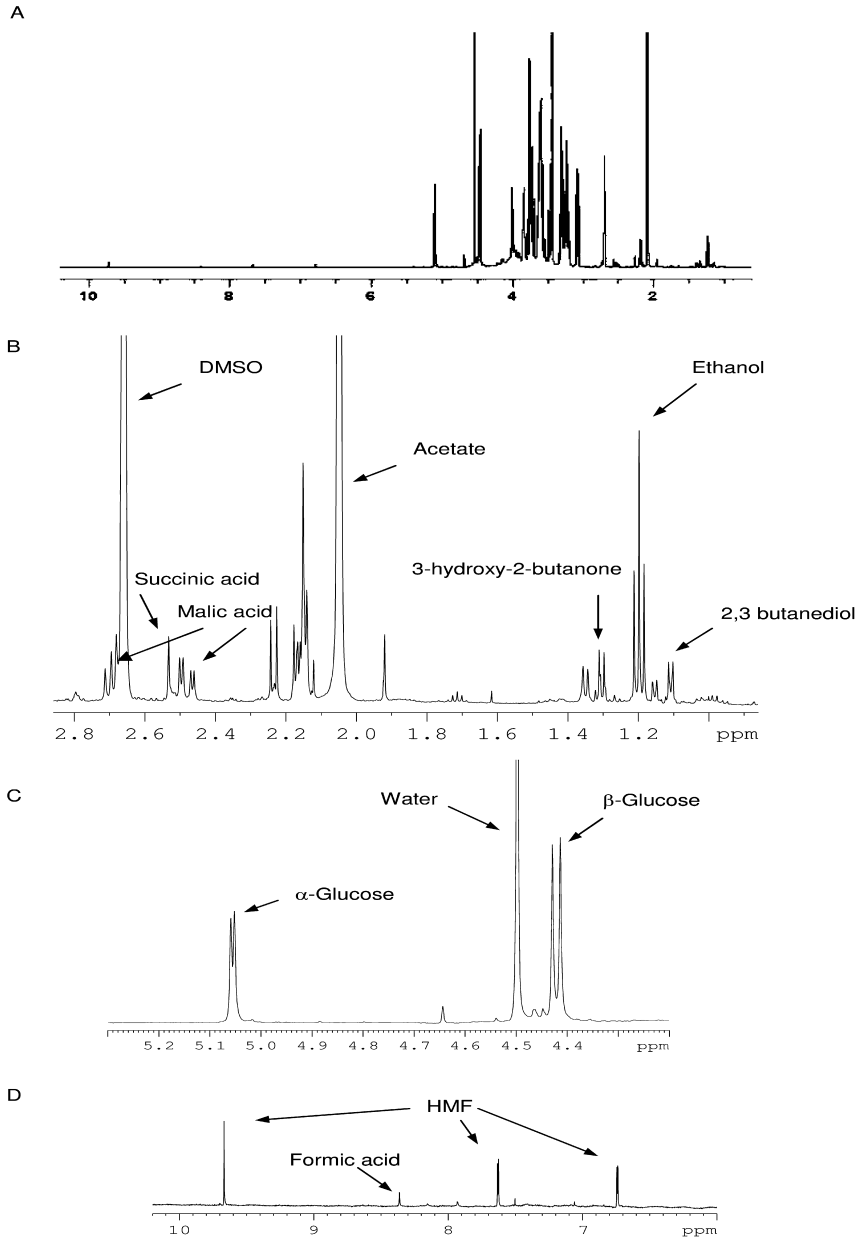

Figure 11. [1]H NMR BVM global spectrum (A) and the aliphatic (B), anomeric (C) and aromatic (D) expansions with principal assignments are reported.

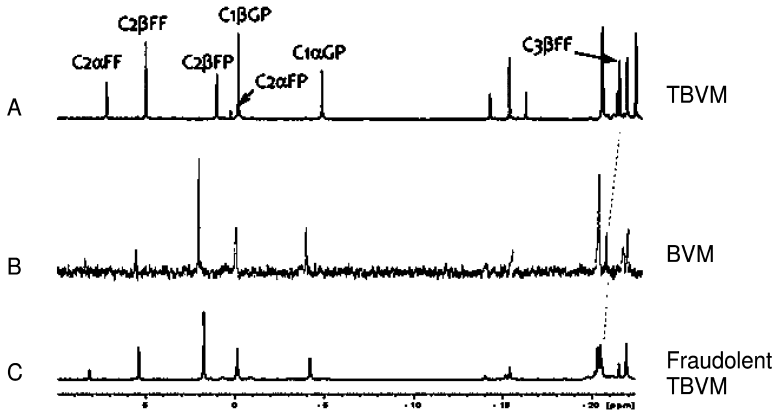

Figure 12. ^{13}C NMR spectra of TBVM (A), BVM (B) and fraudolent TBVM (C) with the fructose and glucose isoform assignments reported.

A further improvement in the knowledge about BVM and TVBM was achieved by recording ^{13}C NMR spectra: the analysis of these data allowed successful TBVM authenticity determination. In this latter study the different isoforms of glucose and fructose were investigated. TBVM samples dissolved into water showed the "natural ratio" of two isoforms for glucose (α,β-pyranosidic) and four isoforms for fructose (α,β-furanosidic and α,β-pyranosidic). When organic solvent was used, differentiation in isoforms ratio of both glucose and fructose was detected. In particular, fructose isoforms ratio reports the preferential degradation of the most abundant β-pyranosidic isoform, well monitored by C2βFP signal, which was present in a lesser extent (Figure 12A). In contrast, completely different isoforms ratio for fructose was detected for BVM samples (Figure12 B); in this case the isoforms ratio results very similar to sugar isoform distribution observed in water solution of sugars or in the must before the cooking process. Monitoring the isoforms ratio and the chemical shift of specific signals of sugars isoforms (Figure 12), three different factors could be used to identify the presence of a fraudolent TBVM and therefore revealed their authenticity: the absence of the α-pyranosidic isoform of fructose, the ratio C2βFP/C2βFF that should be less than 1 and the analysis of the chemical shift deviations for C2 and C3 fructose carbon atoms of α- and β-furanosidic isoforms, respectively, with respect to reference values of highly trusted samples used as reference (*18*).

Conclusions

NMR and chemometric are very powerful in determining the geographical origin and quality of different food matrices. Particularly interesting are the results about the possibility to distinguish food products grown in close proximity but in different soils. This confirms that foods obtained from soil, recorded a sort of "identity" of the place were they come from, thus allowing their recognition. The metabolic determination achieved by NMR spectroscopy was revealed to be very affordable analytical method for approaching food authenticity requirements. On the other hand, chemometrics has a priviledged role in data analysis, expecially in case of the large data matrices obtained by NMR spectroscopy.

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Chapter 3

Establishing Natural Product Content with the Natural Radiocarbon Signature

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Radiocarbon (^{14}C) is produced naturally at relatively constant levels in the atmosphere by cosmic ray interactions with nitrogen. Isolated carbon atoms are quickly oxidized to CO_2 in the atmosphere and are incorporated into biomolecules. Above ground nuclear weapons testing between 1955-63 caused a pulse in the ^{14}C content of the atmosphere over the past 55 years, but it is now almost back to the 1950 level. All living things are labeled with ^{14}C while alive and retain the isotopic signature at death and when processed into food. Carbon from petroleum sources is devoid of ^{14}C and easily distinguished from natural biological sources ($^{14}C/C = 1.2$ parts per trillion) by accelerator mass spectrometry (AMS). We will briefly explain how AMS works and present examples of how it is used to determine natural and fossil carbon content of food. We will also describe how AMS can be used to verify if a claimed vintage is consistent with its isotopic signature.

A natural product is generally thought to be derived from a plant or animal that was recently living. It may have undergone some chemical processing, but we usually view that processing as separatory, isolating specific compounds for use as a flavor, additive or possibly a preservative in a processed food. On the other end of the spectrum are artificial compounds synthesized from petroleum derived precursors. These compounds may occur in nature, but large scale synthesis can be much less expensive than purification from a natural source

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where the desired compound could be in low abundance or difficult to isolate from a complex mixture. Between the extremes of purely bio-derived and petroleum-derived compounds are chemically manipulated natural products. In these a natural product is manipulated to change functional groups or saturate bonds, for example. These products are often low-calorie or poorly absorbed compounds that retain a flavor of a natural product while reducing sugar or fat content. Many processed foods are mixtures of these three types of ingredients.

There are many processed foods that claim to be "all natural" which implies that they consist solely of bio-derived ingredients. All-natural products can often demand a higher price as consumers deem them of superior quality. How can an all-natural product be distinguished from petroleum-derived one if they are chemically identical? Bio-derived carbon and petroleum-derived carbon possess distinct isotopic signatures that can be used to make this distinction.

Radiocarbon analysis is traditionally utilized as a tool for archeological dating (*1*) rather than natural product authentication. Radiocarbon or carbon-14 (^{14}C) is produced naturally in the atmosphere by cosmic ray interactions with nitrogen-14. Single carbon atoms in the atmosphere are chemically reactive and are quickly oxidized to carbon dioxide (CO_2). The CO_2 from the atmosphere is incorporated into plants and works its way up the food chain to label every living thing with ^{14}C. All living things reflect the isotopic signatures of their food sources. The natural atmospheric concentration of $^{14}C/C$ has remained relatively stable at about 1.2 parts per trillion over the past three thousand years (*2*). Anthropogenic activities over the past century are shifting the atmospheric $^{14}C/C$ concentration. Burning fossil fuels increased the concentration of CO_2 in the atmosphere with ^{14}C-free carbon. ^{14}C has a radioactive half-life of 5730 years, so fossil fuel derived carbon that has been dead for millions of years is ^{14}C-free. The slow decline in atmospheric $^{14}C/C$ observed in the first half of the 20th century is known as the Seuss Effect (*3*).

Atmospheric testing of nuclear weapons during the 1950s and early 1960s almost doubled the concentration of $^{14}C/C$ in the atmosphere (Figure 1) (*4*, *5*). From the peak in 1963, the level of $^{14}CO_2$ has decreased with a mean life of about 16 years, not due to radioactive decay, but due to mixing with large marine and terrestrial carbon reservoirs. The ^{14}C has not actually disappeared, it has simply moved out of the atmosphere. In recent years the decline of atmospheric $^{14}C/C$ is believed to be driven more by CO_2 addition from combustion of fossil fuels than sequestration of elevated $^{14}CO_2$ into carbon reservoirs. The temporal variations of artificially high levels of atmospheric radiocarbon have been captured in organic material world-wide and provide a means to determine a date of synthesis for biomolecules. Since radiocarbon is incorporated into all living things, this pulse is an isotopic chronometer of the past 55 years.

The atmospheric $^{14}CO_2$ curve depicted in Figure 1 is a Northern Hemisphere annual growing season average. It is constructed using several independent data sets that used tree rings, recent plant growth, and direct atmospheric sampling to provide carbon samples (*6–10*). Since there were relatively few geographic sources of bomb-pulse ^{14}C, the upswing and the peak values of the curve do vary with geography (*8*, *9*, *12*). However, since CO_2 is a gas and did not fall out of the atmosphere like particulates, the pulse of $^{14}CO_2$ mixed in the atmosphere with all

other CO_2 to produce a relatively homogeneous distribution of atmospheric $^{14}CO_2$ by about 1970 (*9*).

The isotopic content of new plant growth reflects the atmospheric $^{14}C/C$ concentration. Isotope fractionation effects observed in$^{13}C/C$ with C3 and C4 plants are also seen in $^{14}C/C$, so these corrections need to be included. About 1.1% of carbon is the stable isotope ^{13}C. Plants, to a variable degree, can discriminate between ^{12}C and ^{13}C, resulting in differences in the levels of this isotope between different types of plants (*13*, *14*). Furthermore, microclimate variations and environmental stresses can shift ^{13}C fractionation in a particular plant (*14*). Differences in the fixation of CO_2 during photosynthesis distinguish the more common C3 plants from C4 plants. C4 plants have a double fixation step for CO_2 and their photosynthetic pathway is located deeper in the leaves. Isotope fractionation in C4 plants is primarily limited by diffusion (*13*, *14*). This is in contrast to C3 plants which can better discriminate between these isotopes and both reduce the binding of ^{13}C and more readily make ^{13}C diffuse out through the stomatal pores to the outer atmosphere (*13*). As a result, C4 plants (which include corn and sugar cane) contain higher amounts of ^{13}C than C3 plants (which include potato, sugar beet, and wheat) (*15*). In general, C4 plants tend to grow in hotter or drier climates than C3 plants whose open stomata lose too much water to thrive. New leaves are produced in a matter of weeks while larger fruits and vegetables form over the period of a month or two. Herbivores lag the atmosphere slightly because their primary carbon source is on the order of weeks to months old. Omnivores and carnivores lag the atmosphere further because their carbon sources are further removed.

Within organisms, molecules turn over at different rates so ^{14}C levels can vary between molecules. For example, cellulose is relatively static, DNA incorporates new carbon at cell division, lipids can be stored for extended periods, and most other molecules cycle carbon rapidly. The date of formation of a tissue or specific biomolecule can be estimated from the bomb-curve by considering these lags in incorporation and relating the $^{14}C/C$ concentration with the date. The actual data record is relatively noisy because it consists of biweekly atmospheric grab samples (*8–10*). Most biomocules in foods and additives are naturally produced over a growing season. Constructing a bomb-pulse curve from annual averages of the carbon intake over a growing season smooths the curve and can account for much food chain lag. Caution must be exercised when relating an elevated $^{14}C/C$ measurement to a date of formation because the pulse is double valued. Placing a sample on the ascending or descending side of the pulse can often be accomplished if other information is available, e.g. tannins in red wine are a year older than the ethanol (*12*).

Today nearly all ^{14}C forensic analyses are conducted using accelerator mass spectrometry (AMS). AMS is much faster and generally more precise than decay counting since it separates carbon atoms by mass and counts individual ^{14}C atoms vs. a stable ^{13}C or ^{12}C current and is not constrained to wait for atomic decay. If a sample is large (~1 g carbon), readily soluble in liquid scintillation cocktail and does not cause quenching, scintillation counting can distinguish between biological and petroleum carbon sources, but may be challenged to distinguish relatively small depressions seen with a petroleum-derived additive in a largely

natural sample. AMS uses smaller samples than decay counting (~1 mg carbon), an important issue when analyzing specific ingredients or compound classes in a sample.

Sample preparation and measurement details vary among AMS facilities, depending on the type of sample to be analyzed and the design of spectrometer. Routine radiocarbon analyses by AMS require about 1 mg carbon. Samples are dried completely and then combusted with excess oxygen to produce CO_2. The CO_2 is purified to remove water vapor, nitrogen, oxides of nitrogen, and oxides of sulphur. It is then reduced to graphite or elemental carbon on metal catalyst, often cobalt or iron powder.

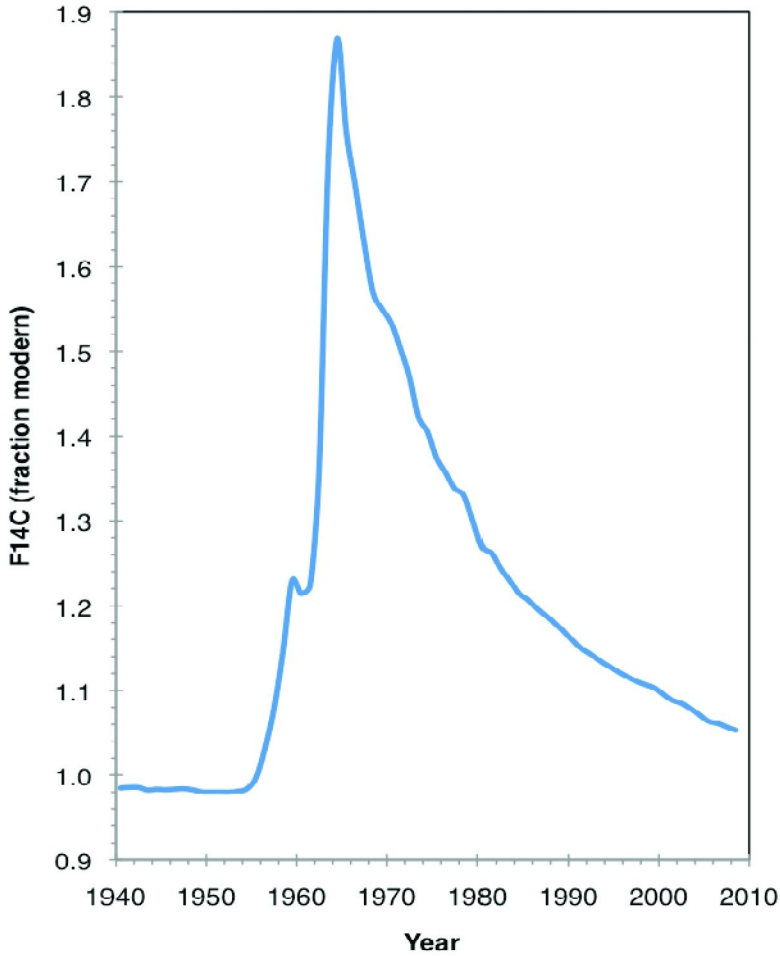

Figure 1. Northern hemisphere growing season average of atmospheric $^{14}C/C$ concentration in CO_2 from 1940-2008. The ^{14}C concentration curve is a compilation of extensive tree ring and atmospheric records (6–10). The $F^{14}C$ (i.e., fraction modern) nomenclaure is designed for expressing bomb-pulse ^{14}C concentrations (11).

Primary standards, secondary standards, backgrounds, and samples under analysis are similarly processed to produce graphite, which is the form of carbon analyzed by the majority of AMS systems. Graphite is the preferred form of carbon because it can be made easily at high purity, produces intense negative ion currents, has very low vapor pressure (which produces minimal sample memory and allows fast switching of samples), and can be prepared at satellite labs and shipped to AMS facilities for analysis. A handful of gas accepting ion sources that take direct feed of CO_2 exist, but they are not widely used for high precision dating. Work is currently underway to develop an ion source interface that could accept a HPLC eluent and directly oxidize it to CO_2 for direct injection into a spectrometer. It is important to have consistent sample source material (e.g., all carbon graphite or CO_2) because different molecules ionize with different efficiencies. Using a single sample matrix for analyses eliminates the need to normalize for variable ionization efficiencies. Methods for producing graphite that were designed for elevated biological tracing experiments (*16*, *17*) can be used for natural product evaluation if exceptional care is taken to carefully determine backgrounds and the associated uncertainties (*18*).

Materials and Methods

Samples

All samples analyzed for natural carbon content were purchased in San Francisco Bay area grocery stores between 2007 and 2010. Products were selected to include a variety of processed foods containing natural and artificial sweeteners and flavorings. The foods could be separated into three general groups: baked goods, cereals, sauces and soft drinks consisting primarily of grains, sugars, and oils; powders requiring water for reconstitution; and specific flavorings.

Sample Preparation

All samples were pepared in the Natural Radiocarbon Prep Lab at Lawrence Livermore National Laboratory (LLNL). Sample prep varied with sample type due to liquid content. Since AMS measures milligram-sized samples, excess water needs to be removed prior to combustion to CO_2. If water is not removed, it becomes water vapor during heating, creates high pressure and causes combustion tubes to explode in the furnace.

Dry powders and bakery products were weighed (2-5 mg) and added directly to combustion tubes. Liquid samples were added to combustion tubes and then dried over 2 days in a convection oven set at 95°C. Ethanol in the flavorings evaporated quickly but water in soft drinks took longer to evaporate. Excess copper oxide (CuO) was added to each dry sample, and the tubes were brought to vacuum and sealed with a H_2/O_2 torch. Tubes were placed in a furnace set at 900°C for 3.5 h to combust all carbon to CO_2. The evolved CO_2 was purified, trapped, and reduced to graphite in the presence of iron catalyst in individual reactors (*19*, *20*). Graphite samples were pressed into aluminum sample holder cathodes and carried to the spectrometer for analysis.

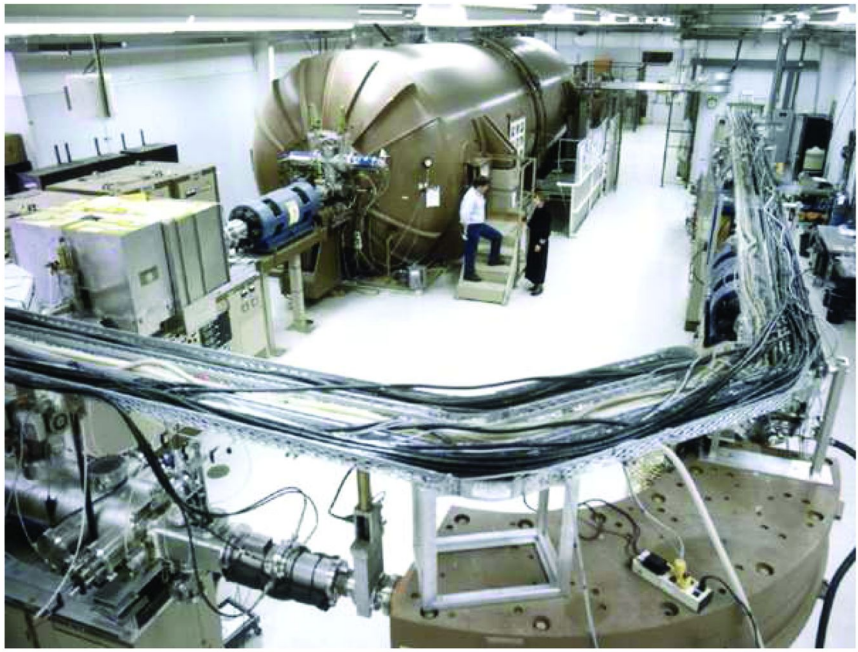

Figure 2. HVEE-FN AMS system at LLNL.

AMS Analysis

All $^{14}C/C$ measurements were completed with graphite targets analyzed at the Center for Accelerator Mass Spectrometry at LLNL on the HVEE FN-class tandem electrostatic AMS system (Figure 2). The operation was similar to that when performing high-precision measurements of dental enamel (*21*). The system employs a LLNL designed high-output negative ion solid graphite Cs-sputter source (*22*) which emits 250-350 μA of $^{12}C^-$ from a full-sized sample, corresponding to approx. 1000 ^{14}C counts per second from a contemporary sample. The FN AMS system routinely achieves 15 % total system efficiency for C analyzing $^{14}C^{4+}$ in the detector (*23*). Details on the design of the LLNL AMS system and its operation can be found in the literature (*22–25*). Natural product investigation samples are usually full sized and contemporary, so analysis times are relatively rapid, generally less than 5 minutes. Samples were measured for 30,000 ^{14}C counts per cycle for 4-7 cycle repetitions and achieved standard deviations of 0.2-1.5%.

A $\delta^{13}C$ fractionation correction of –25±2 or –15±2 was used for all samples based on the dominant carbon source for each sample (C3 or C4 plant). Corrections for background contamination introduced during sample preparation were made following standard procedures (*26*). All data were normalized with six identically prepared NIST SRM 4990B (Oxalic Acid I) standards. Isotopic ssecondary standards NIST SRM 4990C (Oxalic Acid II), IAEA C-6 (ANU sucrose), and TIRI wood B (*27*) were used as quality control samples to monitor

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spectrometer performance. ^{14}C-free coal served as background material for processing the samples. Samples were organized in groups of 12 or 14 unknowns bracketed by primary standards with one primary standard in the middle of the group. The secondary standards, primary standards and group of unknowns are measured consecutively as a cycle. The set of standards and unknown samples are measured repeatedly until desired precision is achieved. A typical group of 14 natural product samples was measured completely in 2-3 h. All ^{14}C data are reported using the $F^{14}C$ fraction modern nomenclature developed for post-bomb data (*11*). $F^{14}C$ is a concentration unit ($^{14}C/C$) denoting enrichment or depletion of ^{14}C relative to oxalic acid standard normalized for isotope fractionation. $F^{14}C$=1 modern can be thought of as the natural atmospheric concentration of $^{14}C/C$ before anthropogenic activities started to influence the ratio. It is actually derived from multi-decadal average of $^{14}C/C$ from pre- and early industrial revolution tree rings from the 19th century (*28*).

Results and Discussion

The natural products purchased between 2007 and 2010 should contain carbon from 2005 to 2009 growing seasons. Terrestrial natural products in this survey should possess $F^{14}C$ = 1.03-1.07. The results of the measurements and assessment of the origin of the carbon are listed in Tables 1, 2, and 3.

Table 1 contains $^{14}C/C$ results for baked goods, cereals, sauces and soft drinks consisting primarily of grains, sugars, and oils. These products tend to posess a natural $^{14}C/C$ signature, even if highly processed. It is possible that some components of a processed food are a year or two older, but generally food products are cycled and not stored for multiple years. The tonkatsu sauce and one cake filling in our survey possessed a higher than expected $^{14}C/C$ value indicative of being produced around 2002-2004. The diet cola with artificial sweetener possessed an isotope ratio indicative of a mix of carbon from natural and petroleum sources.

The powdered and dried foods in Table 2 possessed a mix of natural and artificial ingredients. Products with $^{14}C/C$ values within 5% of the natural range ($F^{14}C$ = 0.98-1.03) were described as almost natural. Those foods with $^{14}C/C$ values more than 5% below the natural range were described as aritificial. Both artificial sweetener samples possessed natural $^{14}C/C$ signatures. Both were produced by manipulating natural products. All powdered drink mixes in the survey contained artificial sweeteners. Artificial sweeteners did not dominate the ingredients, however, and different flavor formulations produced different isotope signatures. Formulations vary because the different flavors possess different solubilities in water. In some cases emulsifiers are needed to keep flavorings in the aqueous phase. The flavored gelatin contained sugar as the sweetener but its $^{14}C/C$ signature was significantly below natural.

Table 1. Baked goods, cereals, sauces and soft drinks

Description	*$F^{14}C$*	*Assessment*
Processed bakery (filling 1)	1.090±0.004	Natural
Processed bakery (cake 1)	1.061±0.004	Natural
Processed bakery (frosting 1)	1.052±0.004	Natural
Processed bakery (filling 2)	1.048±0.004	Natural
Processed bakery (cake 2)	1.054±0.004	Natural
Processed bakery (frosting 2)	1.041±0.004	Natural
Processed bakery (filling 3)	1.062±0.004	Natural
Processed bakery (cake 3)	1.055±0.004	Natural
Breakfast cereal	1.050±0.004	Natural
Cola	1.054±0.004	Natural
Diet Cola	0.799±0.004	Artificial
Marshmallow	1.057±0.004	Natural
Gummy fruit snack	1.053±0.003	Natural
Hoisin sauce	1.043±0.004	Natural
Tonkatsu sauce	1.090±0.004	Natural

Table 2. Dried or powdered foods

Description	*$F^{14}C$*	*Assessment*
Non-sugar powdered drink mix (strawberry)	0.965±0.003	Artificial
Non-sugar powdered drink mix (lemonade 1)	1.071±0.004	Natural
Non-sugar powdered drink mix (lemonade 2)	1.023±0.004	Almost natural
Non-sugar powdered drink mix (cherry)	0.936±0.004	Artificial
Flavored gelatin (powder)	0.769±0.003	Artificial
Artificial sweetener (powder)	1.048±0.004	Natural
Artificial sweetener (powder, 2002)	1.105±0.004	Natural
Gravy mix (powder)	1.076±0.004	Natural
Ramen spice pack (powder)	1.047±0.004	Natural
Children's vitamin (dry)	1.000±0.004	Almost natural

Table 3. Flavorings

Description	*$F^{14}C$*	*Assessment*
Real vanilla	1.059±0.004	Natural
Imitation vanilla	0.038±0.001	Artificial
Lemon extract (pure)	0.983±0.004	Almost natural
Orange extract (pure)	0.979±0.004	Almost natural
Raspberry extract (real)	0.114±0.001	Artificial
Almond extract (pure)	1.000±0.021	Almost natural
Imitation brandy	0.647±0.002	Artificial

The flavorings reported in Table 3 are extracts from specific plants or are clearly identified as imitation. All of these flavorings contained high levels of ethanol which was removed by evaporation. The imitation flavorings surveyed were comprised of predominantly fossil carbon. Unlike extracts that contain hundreds of compounds, the inmitation flavorings contain only a couple synthesized compounds to mimick the natural flavoring. The extracts with almost natural $^{14}C/C$ ratios were sold as "pure" extracts of lemon, orange and almond. They contained 30-80% ethanol, water, and a small amount of extracted oil of lemon, orange and almond. After evaporation of the ethanol and water, small oil droplets coated the quartz combustion tubes. The small depression from natural $^{14}C/C$ was likely due to slight retention of solvents used in the extraction of the oils. The raspberry extract had an unexpectedly low $^{14}C/C$ ratio. It was sold as a "real" extract rather than a "pure" extract. It had less ethanol than the other extracts (~20%), but propylene glycol was a significant ingredient. Propylene glycol is generally made from peteroleum sources and is a common emulsifier in pharmaceuticals, foods, cosmetics, food colors, and flavorings. The carbon inventory of the raspberry extract was dominated by the propylene glycol rather than the natural components.

The precision of radiocarbon dating depends on the ability to measure the ^{14}C concentration in a sample and characteristics of the calibration curve. The slope of the atmospheric ^{14}C curve varies dramatically over time and the precsion of the curve is also variable. It is relatively easy to achieve 0.3-0.5% measurement precision of $^{14}C/C$ when analyzing full-sized samples (>300 μg C) by AMS. For the given measurement precision, the corresponding chronological range is shorter when the curve is steep (1980) than when it is shallow (2010).

The atmospheric $^{14}C/C$ concentration depicted in Figure 1 is not an infinitesimally thin line - it contains uncertainty too. Figure 1 is constructed from published data for clean air collections in a limited number of locations (*6–10*). Extrapolating the published record for clean air collections to agricultural production locations introduces uncertainty to the curve. Agricultural products can have local influences which shift the isotopic ratios slightly. These local influences include exposure to polluted air with a slightly depressed $^{14}C/C$ ratio (*29*), variations in soil respiration rates (*13*), weather conditions which resist

flushing of regions with clean air (*29*), and climatic stresses that effect isotope fractionation (*13*). Since we seldom have a local atmospheric $^{14}C/C$ calibration curve, we need to treat the atmospheric record as a concentration band and include uncertainty in it as well.

Sample size also has an influence on precision. Processing a carbonaceous material into graphite for $^{14}C/C$ analysis introduces both contemporary and fossil carbon backgrounds into the sample (*26*). These background components are established by measuring isotopic standards and ^{14}C-free material over the sample mass range and comparing the measured values to certified values. The contemporary and fossil carbon backgrounds are on the order of 0.8±0.4 μg carbon at LLNL. The uncertainties in these background components have little effect on full-sized samples, but significantly contribute to the uncertainties for samples containing <40 μg carbon. For example, the pure almond extract sample contained only 21 μg carbon and its uncertainty is on the order of 2% compared to ~0.4% for the samples containing ~1 mg carbon.

Conclusions

Analyses of $^{14}C/C$ concentrations in foods, flavorings, and health products can be used to determine natural product content. Natural products consist of bio-derived carbon with a $^{14}C/C$ concentration of about 1.2 parts per trillion. Artificial ingredients produced from petroleum-derived carbon are devoid of ^{14}C. The recent clean air atmospheric record of $^{14}C/C$ in the Northern Hemisphere is data rich for middle and upper latitudes, providing a reliable record of natural product $^{14}C/C$ concentrations. All-natural products should contain a $^{14}C/C$ signature consistent with the atmospheric record over the past 1-3 years. If a measured $^{14}C/C$ concentration is depressed, the product generally contains ingredients synthesized from petroleum-derived carbon.

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Chapter 4

Using Chemometrics To Classify Samples and Detect Misrepresentation

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Multivariate pattern recognition offers a number of advantages in detecting adulteration or misrepresentation of foods and beverages or their ingredients. Since multiple properties are used to make classifications, multiple adjustments would be necessary to perpetrate a successful fraud, which would likely make it uneconomical. Pattern recognition procedures can be either unsupervised (depending only on the structure of the entire data set) or supervised (using the presumed sample class identifications to establish classification rules). Unsupervised methods include principal components analysis (PCA) and cluster analysis. Supervised methods include nearest neighbor analysis, discriminant analysis, SIMCA and partial least squares discriminant analysis (PLS-DA) among others. The degree of success of classification rules produced should be tested by some validation procedure. Examples of pattern recognition applied to beer brands and hop cultivars are given in detail. Other applications are reviewed.

Keywords: multivariate pattern recognition; classification; fraud

Whenever there is economic advantage to adulterate or misrepresent a food, beverage or ingredient thereof, there is incentive to cheat. **Adulteration** is defined as "Addition of an impure, cheap, or unnecessary ingredient to cheat, cheapen, or falsify a preparation; in legal terminology, incorrect labeling,

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including dosage not in accordance with the labels" (The Free Dictionary (www.thefreedictionary.com)). **Misrepresentation** can occur by complete or partial substitution of a (usually) cheaper ingredient for a more expensive or more highly prized one. There can also be accidental misrepresentation due to errors of identification of a material.

Chemometrics is the application of statistical and mathematical methods as well as the principles of measurement science to efficiently extract useful information from chemical data (*1*). One of the major topics in chemometrics is **pattern recognition** (*2*). When two or more observations (measurements) are made on each member of a set of samples, patterns often result that can be used to classify or characterize certain groups of samples. An observation may be the result of a single chemical or physical measurement procedure applied to a set of samples or may be one of multiple results from a method that provides them (such as multiple peaks in chromatography, absorbances at multiple wavelengths in spectroscopy or multiple bands in electrophoresis). Observations may also be genetic (e.g. nucleic acid based) or biological (e.g. morphologically based). A data set for pattern recognition (PARC) can contain observations from any or all of the above.

Each measurement is effectively a different dimension and often measurement results are viewed as coordinates in multidimensional space. Humans are quite good at perceiving structure (such as groupings or separation of samples) in low dimensional space (two or three dimensions). Perceiving structure in more dimensions usually requires methods for reducing dimensionality or perceiving similarity; that is how multivariate pattern recognition operates.

Pattern recognition can be applied to samples in order to classify them in different ways. Agricultural materials may be classified by species, cultivar, growing region or crop year. Processed products may be classified by type, brand or even processing plant. It is possible to classify a single set of samples in different ways. For example, samples of an agricultural material might be classified either by cultivar or by growing region.

Adulterating in a way that simultaneously satisfies multiple criteria is likely to be expensive in terms of analytical chemistry and the purchase of the multiple additives needed to produce a fraud that simultaneously fits multiple criteria. If the possibility of detection is known to a potential cheater, this may provide a disincentive that is adequate to discourage fraudulent behavior. There is clearly greater incentive to cheat when there is a large difference between the value of a product and the cost of the materials needed to perpetuate a fraud.

Pattern Recognition Data Sets

Data sets for pattern recognition are typically organized with samples as rows and measurements (observations) as columns. Note that some analytical procedures produce multiple observations (e.g. multiple peaks in chromatography or absorbances at multiple wavelengths in spectroscopy). For purposes of this discussion each separate observation is considered to be a different measurement. When the number of measurements is substantially less than the number of

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samples, essentially any of the PARC methods can be used. When there are more measurements than samples, the system is '**over-determined**' (*3*); this is problematic for some of the PARC procedures. In some cases a set of samples may not be sufficiently diverse in information content, even when the number of samples exceeds the number of measurements. This can happen when many of the measurements are highly correlated (and thus redundant in information content). Use of stepwise procedures or of subsets of the measurements can often overcome this difficulty. A stepwise method tests the classification utility of each individual measurement to find the 'best' one. Each remaining measurement is then evaluated combined with the one first selected in order to pick the 'best' combination of two measurements. This process is continued until some stopping point is reached (often minimal classification improvement upon including any of the remaining measurements) or before the number of measurements used exceeds the number of samples. With some stepwise procedures, elimination of each previously selected measurement is tested to see if that would improve the result (by reducing error) after each parameter selection step. Ideally selection of measurements chooses a subset with useful information for classification and omits those that contribute mainly noise and redundancy (*4*, *5*). Another approach to overdetermination is to use principal components analysis; as will be shown later, this reduces redundancy and noise and improves the effective sample/measurement ratio.

Missing data present a problem for most pattern recognition procedures. A number of approaches have been used. The simplest and least biased is to not use either a sample or a measurement that is missing. The risk here is that an omitted measurement might be useful for classification. A number of approaches are based on estimating likely values for missing data; this is called **imputation** (*6*). One such approach is simply to replace a missing datum with the average of the column of measurements containing the missing item (*7*). Another is to generate a random number in the range of the data in the column. More sophisticated approaches find 'similar' samples in the data set and use the average of their values for the missing measurement in place of the missing one (*6*).

Many of the PARC procedures can be carried out with the data either in the units in which measurements were made or after some type of scaling. Autoscaling of each measurement column (applying the z-transformation) is frequently carried out to remove the effect of measurement units of different magnitudes (*2*, *8*). In z-transformation, the mean and standard deviation of a column of measurements are calculated. The column mean is then subtracted from each individual value and the result in each case is divided by the column standard deviation. The resulting z-transformed values then replace the original values in the data matrix used to carry out the PARC procedure.

Pattern Recognition Methods

Pattern recognition methods are generally thought of as either **unsupervised** or **supervised** (*8*). The distinction here is whether or not the putative sample classifications are used to obtain a separation between the classes. Unsupervised

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methods do not use this information, while supervised methods employ it to develop classification rules of some sort that can then be applied to new samples in order to classify them.

Unsupervised PARC

Unsupervised PARC methods use the characteristics of an entire data set to see if samples belonging to a class tend to group together and separate from other classes. Ideally this is done by reducing dimensionality and removing redundancy. The main unsupervised PARC methods are Principal Components Analysis (PCA) and cluster analysis.

PCA was described by Pearson in 1901 (*9*) and is the most important technique in chemometrics. PCA extracts components that explain maximum variance in the data that is orthogonal to all previously extracted components (*9*, *10*). That means the PCs are at right angles to one another in multidimensional space and completely uncorrelated – each PC represents completely different information from all the other PCs. Measurements that are redundant or opposite in information content are 'loaded' on the same PC in the same or opposite directions, respectively. By its nature, the information content of a PC (expressed as its eigenvalue, a measure of the variance explained) always decreases as additional PCs are extracted. The loading of a measurement onto a PC ranges from -1 to +1 (with autoscaled data) and has the same sense (magnitude and direction) as a correlation coefficient. Loadings with relatively high absolute values have strong relationships to a PC and the PC is often interpreted as representing that property.

Each sample is projected onto each PC, resulting in a single number representing its location along the PC; this is called the PC 'score' on that component.

The number of PCs needed to represent the information content in a data set is often determined in one of several ways (*11*). In one of these, a plot of the eigenvalue versus the component number, called a Scree plot, is examined to see if there is a sharp break or "knuckle", as at PC4 in Figure 1. If so, the number of components corresponding to the break is usually chosen as the 'correct' number of PCs. A second approach is to keep all components with eigenvalues greater than 1 (i.e. better than average explaining power); this is only appropriate when the data have first been standardized. Another approach is the factor indicator function (IND) of Malinowski; this has a minimum at the proper number of factors (*12*). Yet another method is to test components for significance using cross-validation (*13*). In this approach some fixed number of samples is removed from the data set, PCA is carried out without them and the resulting PC model produced is used to estimate the scores of the omitted samples from their measurement values. The differences between the scores computed from the models made with and without the omitted samples (residuals) are noted. The excluded samples are then returned and an equal number of additional samples are removed and the procedure is repeated until each sample has been excluded once. The residuals are used to calculate a statistic that evaluates the significance of each PC; often

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this is the prediction error sum of squares (PRESS). Additional PCs are extracted until a non-significant PC is reached, at which point the process stops and the previous number of PCs is deemed the 'correct' number to capture the information in the data set. With small data sets, the number of samples excluded in each iteration may be one (called leave-one-out cross-validation). With large data sets, the total number of samples is often divided by seven to arrive at the number of samples to exclude in each iteration. Whichever approach is used, the objective is to choose the break point between signal and noise. Many variations on the use of cross-validation for determining the number of significant PCs have been explored (*14*, *15*). Because a set of measurements often contains considerable redundancy, either because of the nature of the methodology or of the nature of the analyte behavior (often some constituents of a sample are produced by the same or competing pathways), frequently many fewer PCs than original measurements can capture the information content of a sample set. The number of 'significant' PCs indicates the number of fundamental properties contained in the data set. Reducing dimensionality greatly aids in plotting and conceptualization.

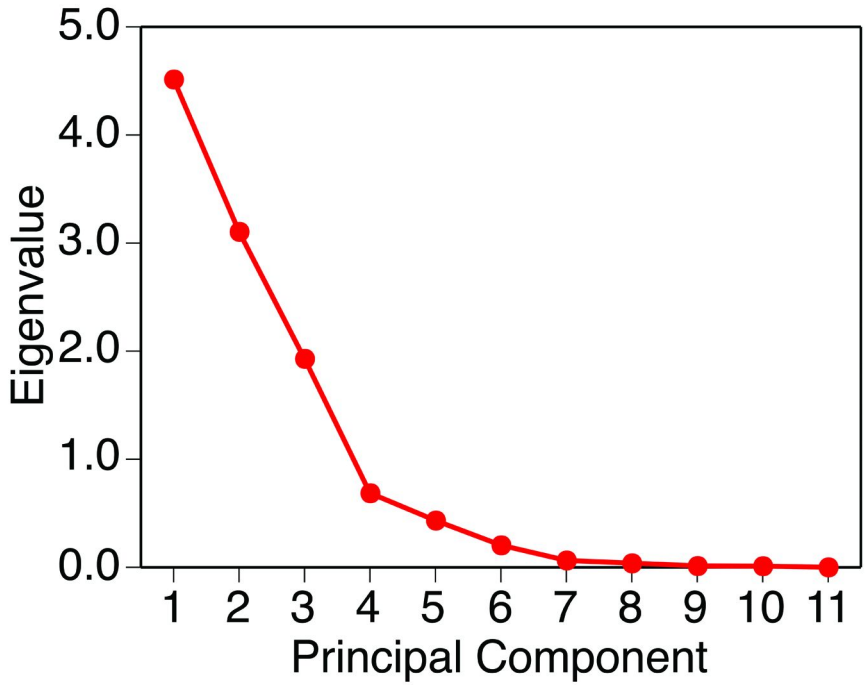

Figure 1. Eigenvalue vs. PC number (Scree) plot demonstrating a clear break at PC4.

There are a number of variations of **cluster analysis**, including hierarchical, non-hierarchical and fuzzy clustering (*8*). Some metric (distance, correlation or a combination of the two) is used to represent the similarity of samples. Most often the measurement values of samples are considered to be coordinates in multidimensional space. The Euclidean (vector) distances between each pair of samples are then computed. That effectively reduces the multidimensional system to a two-dimensional distance matrix. The magnitude of the distance (absolute value) is then used as the metric of similarity. Samples that are close together (having relatively short vector distances) are considered more similar than those that are located further apart. A procedure is then employed to group 'similar' samples together. With hierarchical clustering (see Figure 2), the outcome is often represented in the form of a dendrogram (tree diagram). Hierarchical and non-hierarchical clustering assume that each sample belongs to one and only one class. With fuzzy clustering, a sample can have partial membership in two or more classes, resulting in a more probabilistic representation of class membership (*8*); this has advantages in some cases.

For the unsupervised PARC methods, over-determination is generally not problematic. With PCA, the number of significant PCs is usually considerably fewer than the number of measurements (and often as few as two or three). As a result, the procedure effectively reduces the number of 'measurements', thereby increasing the sample/measurement ratio. And with cluster analysis, the reduction to a distance matrix usually reduces dimensionality even more.

Supervised PARC

Supervised pattern recognition methods use the class identifications of the samples provided by the user to attempt to develop rules or procedures that will separate members of the classes from one another (*8*). There are a host of methods for this. Some of the commonly used approaches are *k*-Nearest Neighbor Analysis (*k*-NN), Discriminant Analysis (DA), SIMCA and Partial Least Squares Discriminant Analysis (PLS-DA). Supervised PARC methods are sometimes separated into those that place boundaries between classes and those that produce class models (*16*). DA makes explicit boundaries between classes while *k*-NN produces implicit boundaries. SIMCA models classes. The samples in the data set comprising a class define the class region in multidimensional space, and this in turn affects the classification rules developed. For example, samples representing a particular cultivar grown in a single crop year may not encompass all of the variation seen in different crop years or vintages of the same cultivar (if that is desired). If, for example, it is desired to define a class as including members of the same cultivar from different crop years, then the samples representing the class must contain samples from multiple years. Similarly, the composition of a finished product may change with storage. It would be wise to include samples of a product that encompass different ages and storage conditions (e.g. temperatures and light exposure) in order to recognize genuine products that have aged.

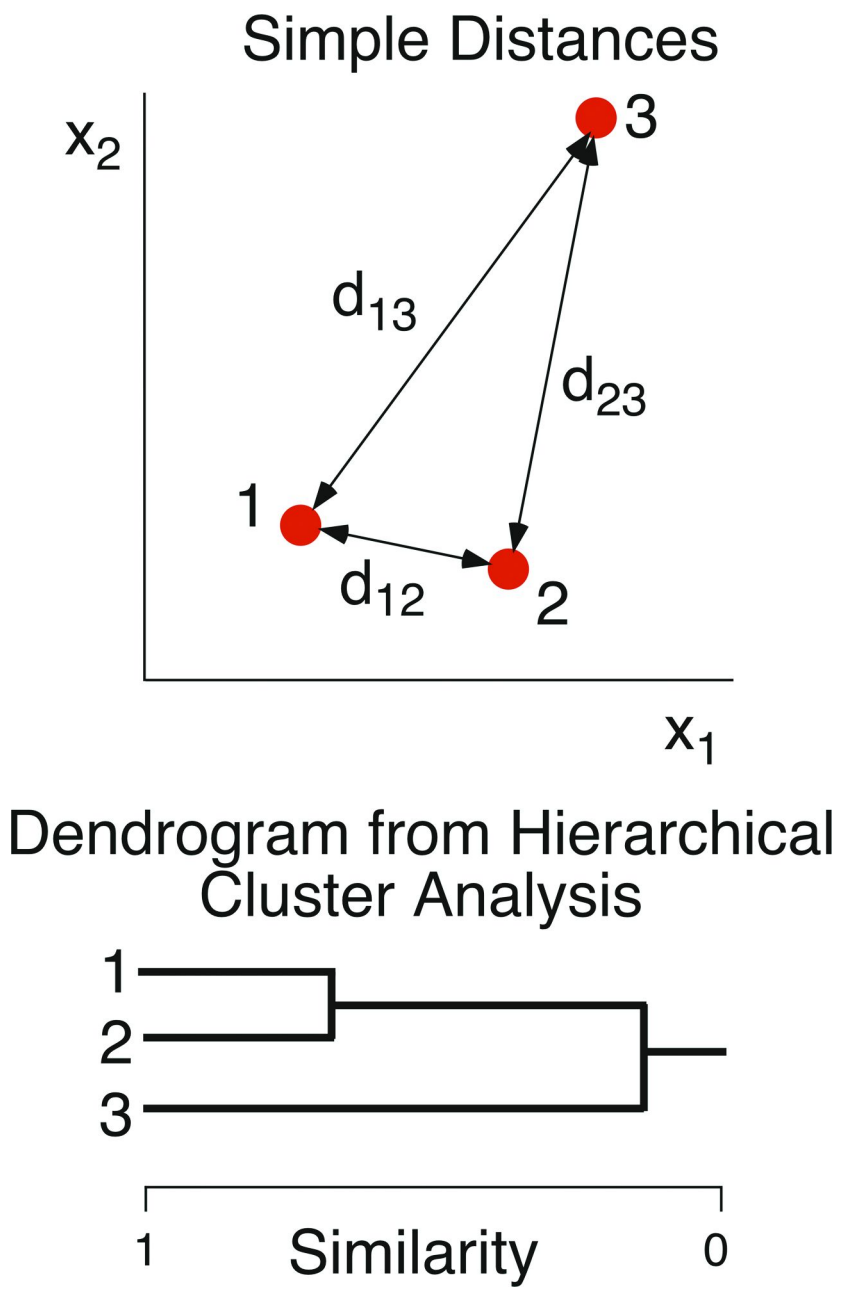

Figure 2. Explanation of hierarchical cluster analysis. The sample measurement values are used as coordinates and the Euclidean (vector) distances between samples are calculated. Samples with relatively short vector distances are assumed to be more similar than those with longer distances. A dendrogram is typically used to represent similarity.

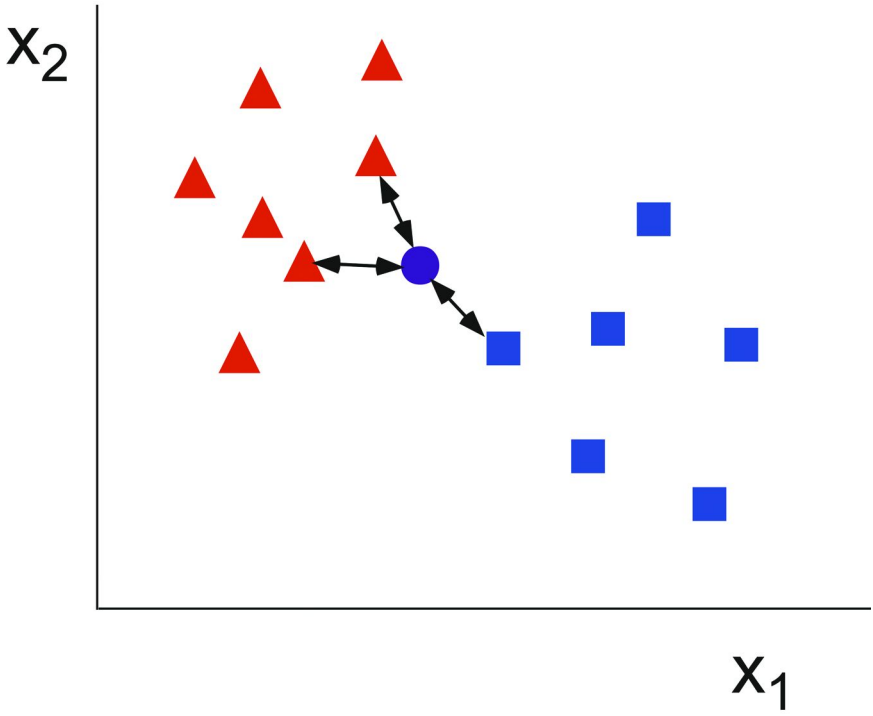

Figure 3. Explanation of k-Nearest Neighbor classification using k = 3. The class identities of the three samples closest to the unknown (round symbol) are noted. The majority of these (two in this case) are represented by red triangles so the unknown would be assigned the class indicated by this symbol.

With *k*-NN, the Euclidean distance metric (as described under cluster analysis) is used (*8*, *17*). Some (usually odd) integer value of *k* is chosen. The class identities of the *k* nearest samples to the unknown are determined (see Figure 3). An unknown sample is classified as belonging to the majority. For example, if *k* = 3 and two of the closest samples to an unknown belong to class B and one to class C, the unknown would be classified as belonging to class B. Often classification is carried out with different values of *k* to find out which works best (sometimes this is evaluated with cross-validation). A disadvantage of *k*-NN is that it cannot provide information about the reliability of classification of particular samples (*2*). If the numbers of samples of different classes are disproportionate, *k*-NN over-assigns unknowns to the class with more numerous samples.

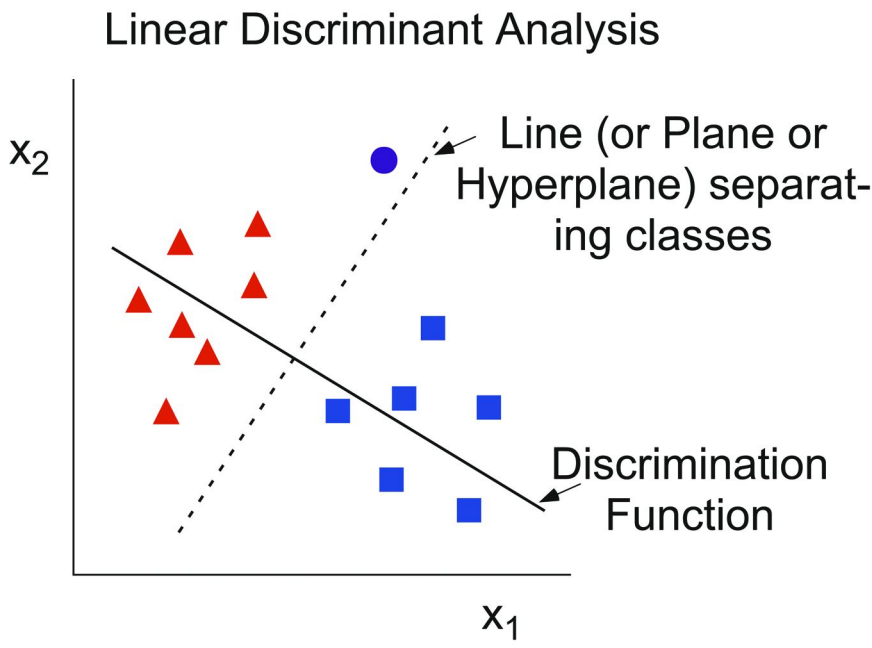

Figure 4. Explanation of linear discriminant analysis. A boundary (in this two dimensional example a line) is constructed that separates each pair of sample classes. An unknown sample (indicated by a round symbol) is classified according to the side of the boundary it falls on (the sample class indicated by triangles).

Discriminant analysis (DA) attempts to calculate boundaries between groups of samples belonging to different classes (*18*). It does this by constructing boundaries between each class pair (*8*). In the case of linear discriminant analysis (LDA) the boundaries separating class medians are linear (lines in two dimensions, planes in three dimensions or hyper-dimensional planes in higher dimensions). If only two measurements are used, this would be a two-dimensional system and lines would be used to separate the classes (see Figure 4). In quadratic discriminant analysis (QDA), the boundaries between classes are curved rather than linear (*19*). Regularized discriminant analysis (RDA) explores a range of possible assumptions about normality of distributions and equivalences of variance or covariance (*20*); these span both LDA and QDA. With hard boundaries, an unknown sample is assigned a classification based on its location relative to the boundary dividing classes, even if it falls far away from the other samples in a class. So discriminant analysis always produces a classification for an unknown sample into one of the previously defined classes. This does not always make sense, as the sample being classified could be from an entirely different class than those used to develop the classification rules.

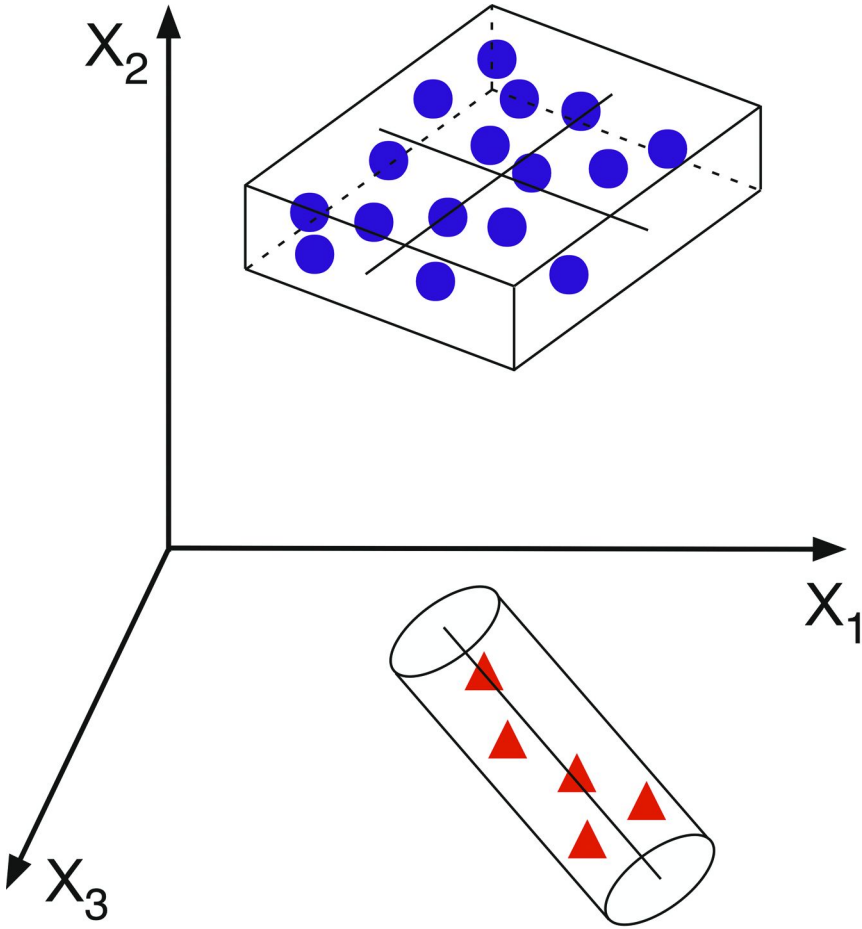

Figure 5. Explanation of SIMCA. Separate PCA models are constructed for each sample class. In each class model each PC calculated is tested for significance by cross-validation until a non-significant component is reached; the number of significant PCs is one fewer. In this example the classes have one (the triangles) or two (the circles) significant PCs.

SIMCA (Soft Independent Modeling of Class Analogy) constructs a separate PCA model for each sample class (*8*, *21*). Cross-validation is used to determine the number of significant components to retain for each class (*13*). When a non-significant PC is reached, the process stops. The number of significant PCs for a class can be 0, 1, 2, 3 or even more. The dispersion of the samples around the resulting centroid, vector, plane or hyperplane is used to enclose the samples in a sphere, cylinder, box or hyperbox, respectively (see Figure 5). If an unknown sample falls inside one of the resulting class envelopes, it is identified as a member of that class. If it falls outside all of the class envelopes, it is considered to be unclassified (a member of a previously undefined class or a hybrid or an outlier). It is also possible for two class envelopes to overlap and for an unknown to fall

into this region, in which case it is considered to be a member of both classes. If no PCs are significant, that indicates that the samples specified as members of a class are not actually homogeneous (i.e. they do not have a consistent pattern). Unlike DA, SIMCA does not make hard boundaries separating classes.

Because SIMCA places boundaries around classes, it can readily detect samples that are outliers in any direction (see Figure 6). This makes it useful for detecting adulteration or for multivariate QA/QC (where the envelope represents samples of good quality).

Partial Least Squares (PLS) is a regression technique often used to model one or more dependent variables as a function of a number of independent variables (*3*). If dummy dependent variables indicating the class identity of samples (each variable having a value of 1 for one class and 0 for all others) are used, the analysis focuses on the measurements that are most useful in separating the classes. This approach is called PLS Discriminant Analysis (**PLS-DA**) (*22*). PLS-DA does not bound sample classes as SIMCA does, but typically outperforms SIMCA when within-class variability is low (*22*).

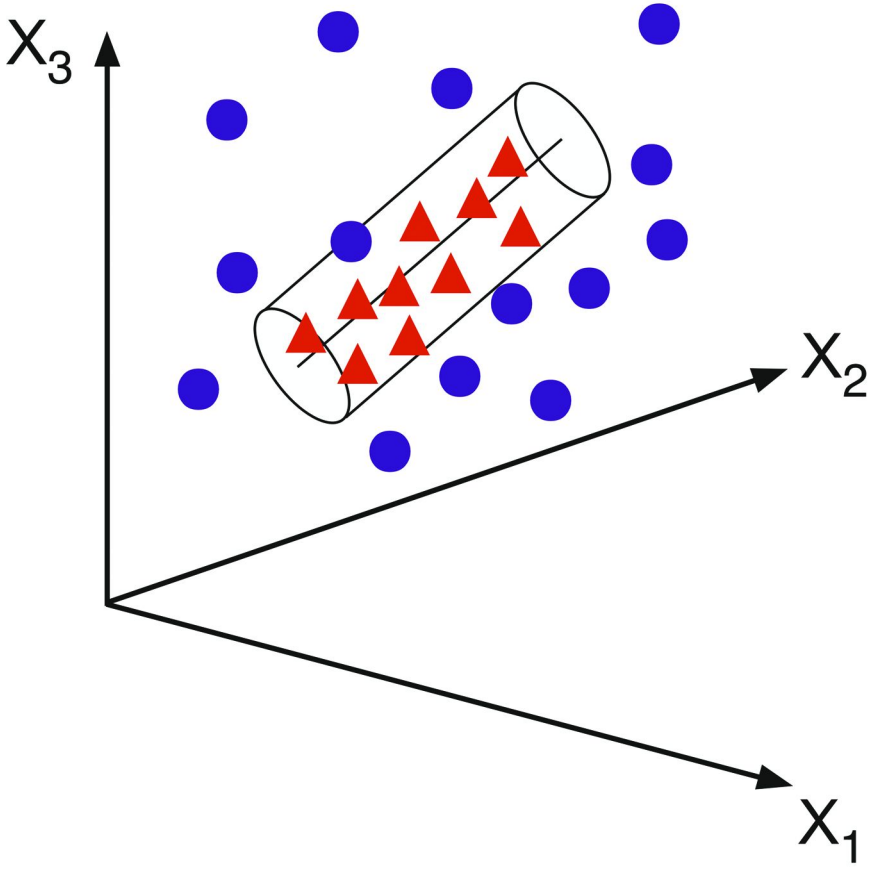

Figure 6. SIMCA model of a class of genuine samples surrounded by outliers in multiple directions.

Artificial neural networks (ANN) are often used for classification (*8*). These model non-linear relationships through multiple layers and connections (nodes). Effectively this means that the user generally does not know the manner in which the various inputs interact and propagate. As a result, it is usually not possible to know which predictors are most important or how they exert their effect (by what non-linear function or interactions). Bruce Kowalski, one of the pioneers of chemometrics, advocated the use of the principle of Occam's Razor in selecting which chemometric procedure to use (*23*). He advocated using the least complex procedure for a task that will successfully model it. Because of their complexity (number of parameters estimated) and the lack of information about how they work, he placed ANN, especially with multiple nodes and layers, as the technique of last resort. Another problem with ANN is that irrelevant measurement variables may cause the network to focus its attention on the idiosyncrasies of individual samples rather than the broader picture of class separation (*2*).

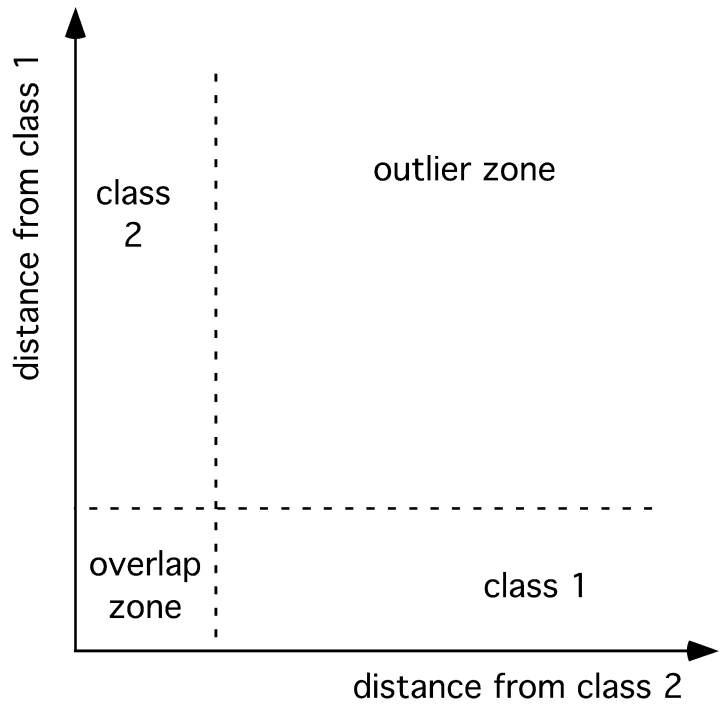

Figure 7. Coomans plot showing distances between two class models. Samples falling below the horizontal dashed line are within the class 1 envelope. Those to the left of the vertical dashed line are within the class 2 envelope. Samples in the overlap zone can be said to belong to both classes while those outside both belong to neither.

Actual Class	Computed Class							
	1	2	3	4	5	6	7	8
1	95.2	0.0	4.8	0.0	0.0	0.0	0.0	0.0
2	0.0	100	0.0	0.0	0.0	0.0	0.0	0.0
3	3.6	0.0	96.4	0.0	0.0	0.0	0.0	0.0
4	0.0	0.0	0.0	100	0.0	0.0	0.0	0.0
5	0.0	0.0	0.0	0.0	100	0.0	0.0	0.0
6	0.0	0.0	4.5	0.0	0.0	81.8	0.0	13.6
7	0.0	0.0	0.0	0.0	0.0	0.0	85.9	15.1
8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	100

Figure 8. Example misclassification matrix (% of samples of a class assigned to a class by a pattern recognition procedure).

Some of the PARC procedures can indicate the distance from the model to a particular sample. This is a probabilistic representation of how well or how confidently the sample is classified. One such approach is a Coomans plot (*24*), see Figure 7, in which the location of samples relative to two different class models is shown. A sample may belong to class 1, class 2, both (the overlap zone) or neither (the outlier zone). The limits are often set at the p = 0.05 level.

Results of supervised PARC procedures that form hard boundaries (e.g. LDA or *k*-NN) are often displayed as misclassification matrices (Figure 8), also called confusion matrices. These indicate which classes are difficult to separate from one another. With a perfect classification, all of the results would fall on the diagonal from top left to bottom right, indicating complete agreement of classification of samples with their putative classes. Misclassification matrices are often prepared separately for training and test data sets. SIMCA results are better represented by Coomans plots than misclassification matrices. However, with multiclass situations forced assignments may be used and in that case misclassification matrices are appropriate. Performance of different PARC methods on a data set can be compared using the % of samples, either in a training or test data set, classified correctly.

The supervised PARC methods vary in their degree of sensitivity to overdetermination. *k*-NN, like cluster analysis, uses a distance matrix to identify the closest samples; this effectively reduces the dimensionality to 2, making overdetermination non-problematic. For LDA, overdetermination is a serious problem. One solution is to carry out the analysis several times, in each case using only a limited number of the measurements (which must be smaller than the number of samples). Most commercial computer programs that carry out PARC procedures give an indication of the relative utility of particular measurements in making classifications. It is often beneficial to remove from the data set (i.e. not use) measurements with limited utility for separating classes. This removes samples that provide more noise than useful signal and often strengthens classifications (reducing PRESS). If LDA is carried out with partial sets of measurements, then the most useful measurements from each subset of the data can be combined, often with beneficial results.

Because SIMCA carries out PCA of each class separately, it improves the sample/measurement ratio by effectively reducing the number of 'measurements' used in the same way as PCA does. PLS-DA also carries out PCA, but steers the choice of PCs toward those that not only explain variance but also separate the dependent variables (in this case by their class identities).

A detailed discussion of the advantages and disadvantages of various supervised PARC procedures was provided by Derde and Massart (*25*). Very briefly, and among many other considerations, non-parametric procedures such as *k*-NN make no assumptions about population distributions and are preferred when this information is unavailable. Outliers negatively affect LDA performance more than other procedures. When classes resemble one another closely, modeling procedures like SIMCA perform better than discriminating procedures.

Validation

An important issue with classification models is testing or 'validation' of the classification rules (*16*). This can be accomplished by either internal or external validation. In external validation, some of the samples representing each class are removed from the data set and the classification rules are produced using the remaining samples (the training data set). The classification rules developed are then applied to the reserved samples (the test data set). The extent of agreement between the assigned and actual identities is used to assess the validity of the rules produced. This is considered to represent the **prediction ability** of the classification.

Internal validation employs the same samples used to derive the classification rules (the training data set) for validation, and tests the ability of the rules produced by the procedure to correctly classify them, often with cross-validation. This is considered to represent the **recognition ability** of the classification (*16*). External validation is a more stringent test and is preferred, but sometimes the number of samples in a data set is too few to permit this.

Examples

Beer Brand Data

A brewing company obtained samples of its own and competitors' products quarterly and carried out a battery of analytical procedures including routine beer analyses (many of these by separate wet chemical procedures), volatile compounds determined by gas chromatography, organic acids by HPLC, etc. A total of 53 items were measured. A data set with nine different beer brands, each sampled 6-8 times several months apart, was obtained. Within this data set there were missing values for six of the measurements (all organic acids); those measurements were dropped, resulting in 47 different observations on each of 62 beers. The data were autoscaled and PCA was then carried out (*26*). The Scree plot did not have a clear break. Seven PCs had eigenvalues greater than 1, indicating that the 47 measurements contained information on only seven fundamental properties. The score plot on the first two PCs is shown in Figure 9; this indicates fairly

good separation of the brands. The same symbol shape indicates products from a single company; the regular beers have solid symbols and the light beers open symbols. It is apparent that in each pair the light beers are displaced to the right and upwards (higher score values on both PC1 and PC2) from the corresponding regular beers. The loading plot on the first two PCs (Figure 10) shows why this is the case. In the same direction as the light beers were displaced is the vector for real degree of fermentation (RDF), which is always higher in light beers. And opposite to this vector are those for carbohydrates, original gravity (OG), real extract (RE) and calories, indicating that light beers are aligned with low values of these properties. The products from different companies mainly differed in their scores on a diagonal from high PC2 & low PC1 to low PC2 and high PC1. This corresponded to isobutanal and color. This unsupervised approach to PARC was quite successful in separating the brands, and was even better when the third PC was included (Figure 11).

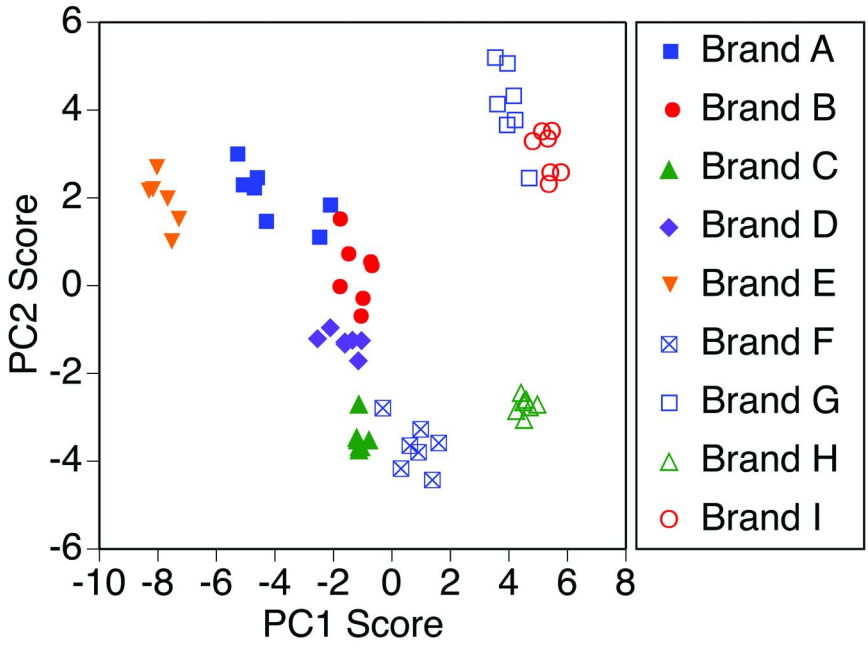

Figure 9. Scores of the samples of the nine brands on the first two PCs. The first and second PCs explained 35.2% and 17.1% of the variance in the data set, respectively. Reprinted with permission from Siebert (26). Copyright 2005 American Society of Brewing Chemists.

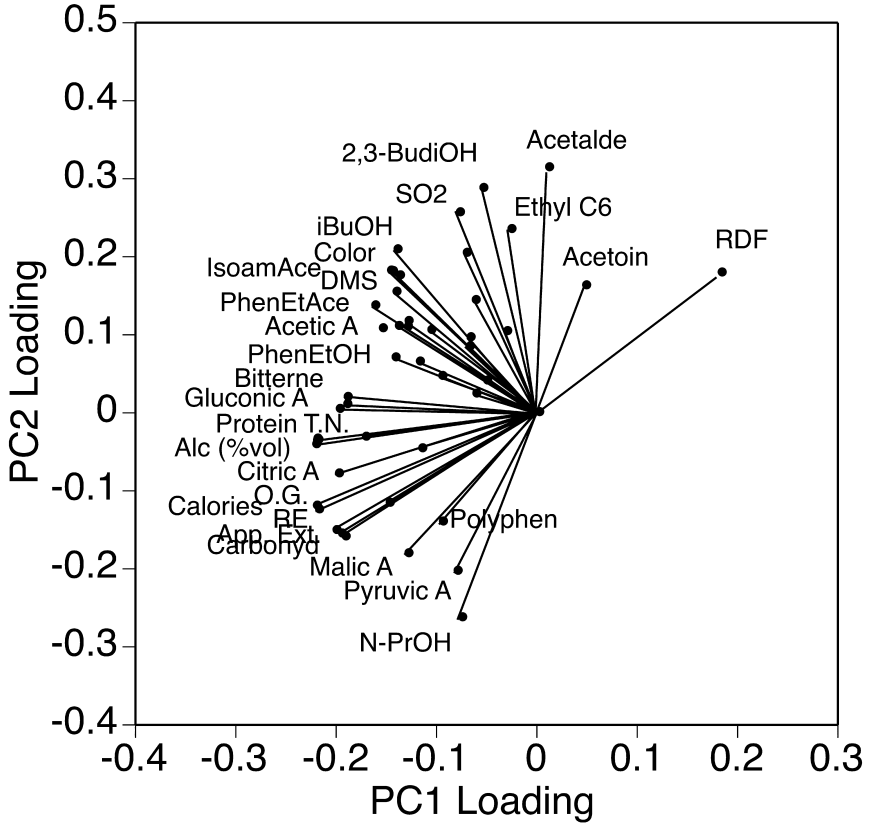

Figure 10. Loadings of the 47 measurements on the first two PCs. Reprinted with permission from Siebert (26). Copyright 2005 American Society of Brewing Chemists.

It was demonstrated that the essential information in the first 8 PCs could largely be captured with only 14 measurements (*26*), and that was by no means an exhaustive attempt to minimize the number. Again the score plot on the first two PCs gave a fairly good separation by brand. As a result, it is clear that the same amount of information could be captured by selecting many fewer measurements, presumably with considerable cost savings. It is likely that many other companies could benefit from examining the collective amount of information in the analytical procedures they employ in order to eliminate redundancy. Naturally, it is economically advantageous to use measurements for PARC that are routinely applied for process control or other purposes, as the analytical cost to obtain these has already been paid (assuming that the results have some utility for classification).

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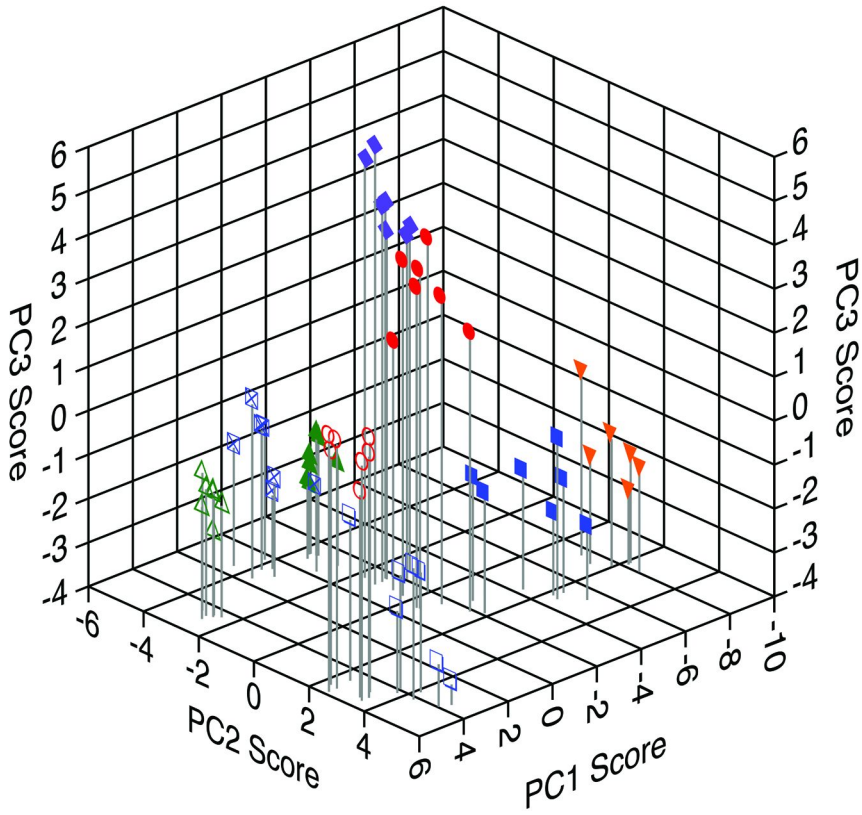

Figure 11. Scores of the samples of the nine brands on the first three PCs. These explained 35.2%, 17.1% and 12.3% of the variance in the data set.

Supervised pattern recognition procedures were applied to the beer brand data (*26*). The classification success of applying the rules obtained to the training set was examined both 'as is' and with cross-validation (see Table I). LDA could not be directly applied to the entire data set because of overdetermination (more measurements than samples). The data set was arbitrarily split into three sets of 22 measurements, representing approximately the first, second and third thirds of the measurements. LDA was 100% successful classifying these subsets of the data as is, but slightly less successful with cross validation. The discriminating powers of each measurement in producing the three LDA subset classifications were examined. Of these, the 11 measurements with the greatest discriminating power were selected and used to again carry out LDA; this led to 100% classification success even with cross-validation.

Table I. Pattern Recognition Procedure Success Rates for Classification of Beer Brands

	Classification Success (%)	
Pattern Recognition Procedure	*As Is*	*Cross Validated*
Linear discriminant analysis		
First 22	100	98.5
Second 22	100	97.0
Last 22	100	97.0
Selected 11	100	100
k-nearest neighbor analysis ($k = 1$)		100
Soft independent modeling of class analogy		
All measured	100	55.2
Selected 17	100	95.5

The computer program used in this study (SCAN) carried out *k*-NN with different integer values for k and used cross-validation to compare their success rates (no 'as is' classification was done). The program judged that results were best with $k = 1$, which produced 100% success with cross-validation.

SIMCA was successful using all of the measurements as is, but was quite poor with cross-validation. This can occur when some of the measurements contribute more noise than information useful to class separation. One statistic produced by the implementation of SIMCA in SCAN is the 'discriminating power' of each measurement. These values were examined; the 17 measurements with the best discriminating power were selected and SIMCA was repeated with these, resulting in 100% success as is and 95.5% success with cross-validation, a considerable improvement.

It is interesting to note that the particular measurements used by SIMCA and LDA in producing their very successful classifications were, in the main, entirely different. Only calories and acetic and citric acids were found in both lists. The particular samples that proved difficult to correctly classify by the two approaches were also entirely different, although they were sampled (and presumably analyzed) on the same two dates. This occurred because of the different manner of operation of the two procedures. LDA constructs boundaries that separate each pair of classes, so measurements that tend to have rather different values for two classes are the most useful. In the case of SIMCA, the most useful measurements are those that define class behavior (representing the major orientation of points belonging to a class in space). Measurements that have different values in different classes are also useful.

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Hop Essential Oil Study

Hops contribute both bitterness and hop aroma to beer and brewers think of different hop cultivars as either 'bitter' hops or 'aroma' hops. Bitter hops have high levels of alpha acids (precursors of the bitter isoalpha acids) and low levels of essential oils, while aroma hops have the opposite pattern. Aroma hops (particularly certain European hop varieties) generally command higher prices and particular varieties or mixtures thereof are used in different beer brands. It was of interest to characterize hop cultivars by their patterns of essential oil compounds. A total of 148 hop samples representing a number of cultivars from each of several crop years were obtained (*27*). The samples were ground and in each case subjected to steam distillation to collect the oil fraction. The resulting samples were subjected to capillary gas chromatography. Peaks were integrated and matched up between chromatograms. There were a total of 117 different peaks, not all of them present in all of the cultivars. Of these, only 25 of the peaks had been identified. Note that it is not necessary to know the identifies of peaks to use them for classification. It is, however, necessary to carefully align peaks between chromatograms. A custom computer program was written to do this as a human would approach it. First large peaks (larger than minimum area values) were sought in fairly wide retention time windows. If sufficiently large peaks were found (not all cultivars had all of these peaks), they were considered to be 'identified'. Based on the location of these identified large peaks within their expected retention time windows, retention expectations were adjusted for intervening peaks. Peaks below a threshold size were ignored.

For the most part, the hop cultivars grown in different parts of the world are entirely different. For example, none of the 'American' cultivars is grown commercially in Europe, and very few of the 'European' cultivars are grown commercially in North America. This set of samples included a few samples of European-type cultivars grown in Idaho. That permitted two ways to attempt classification of the same samples, by actual place of growth (Europe vs. North America) or by European-type vs. North American-type cultivar. Stepwise LDA was carried out with the samples classified both ways (*27*). It was entirely successful in classifying by European-type vs. North American–type cultivar. The procedure showed a small degree of overlap when the samples were classified by the actual place of growth. It was interesting to note that the measurements selected for making the two classifications were mostly different. The compounds selected in the two cases should be informative about the actual place of growth on the one hand and the cultivar type on the other.

Eight cultivars (7 European and 1 North American) were well represented in the data set. Data from these was subjected to PARC by several methods (*27*). *k*-NN was carried out with several values of k. The best results were obtained with $k = 1$. The samples of one of the classes were classified completely successfully. Some samples of all of the other classes were misidentified, with an overall success rate of 76.7% (see Table II).

LDA was slightly more successful than *k*-NN, with an overall success rate of 80%.

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Table II. Pattern Recognition Procedure Success Rates for Classification of Cultivars Using Hop Essential Oil GC Results

Method	*% Success*
Linear discriminant analysis	80
k -Nearest-neighbor analysis	77
SIMCA	100

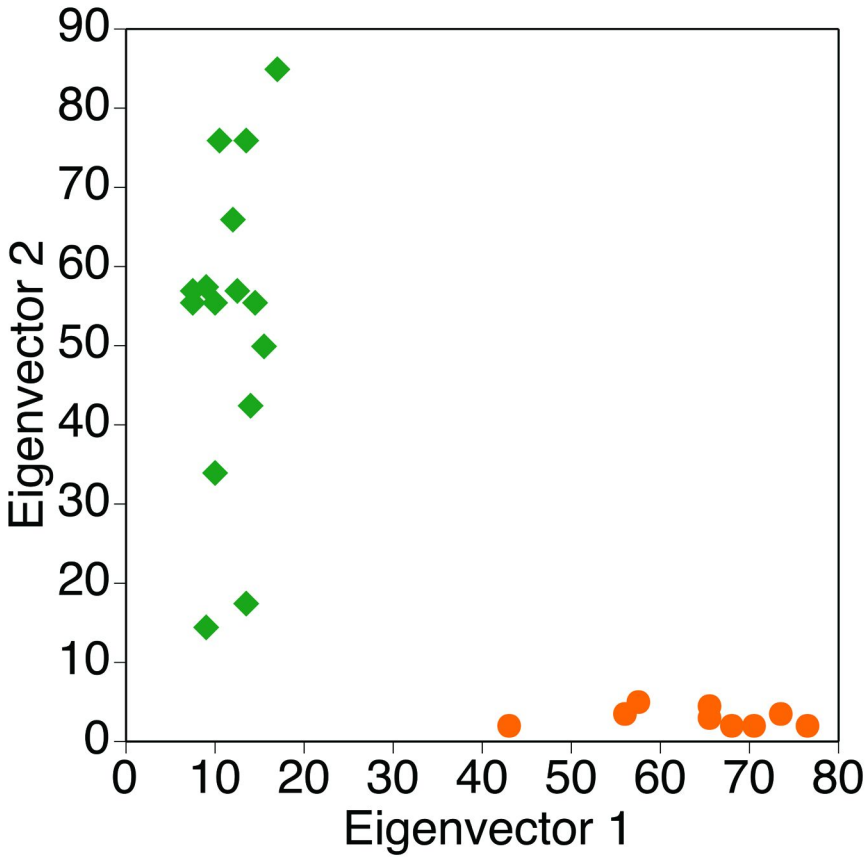

Figure 12. Coomans plot of SIMCA classifications of the European hop variety Spalt (F) and the American variety Clusters (J). Reprinted with permission from Stenroos and Siebert (27). Copyright 1984 American Society of Brewing Chemists.

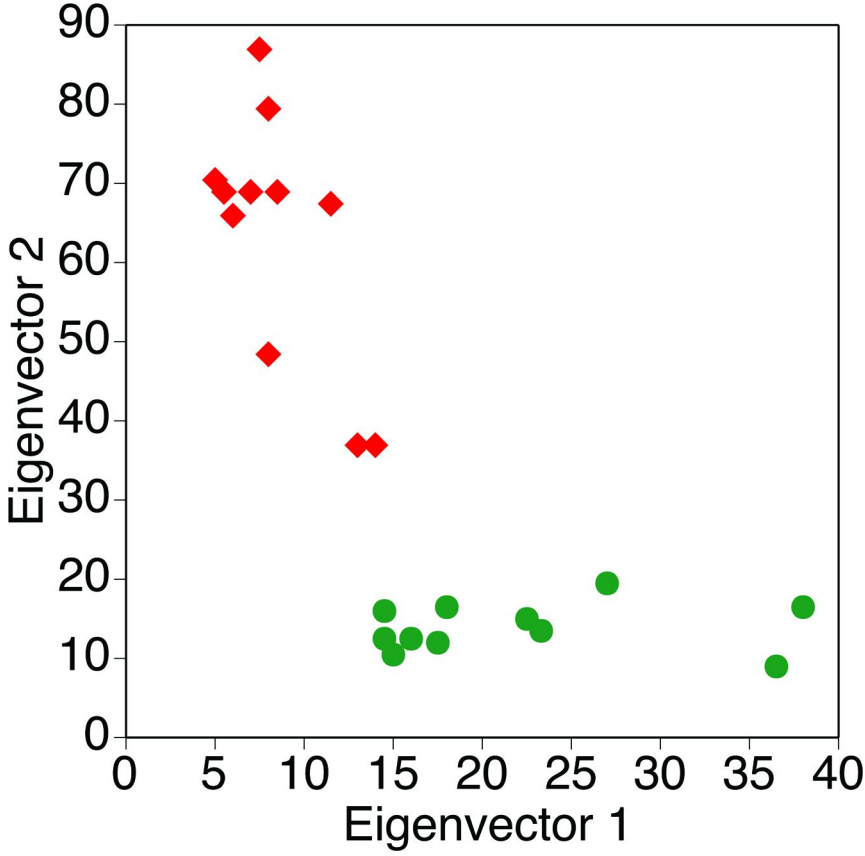

Figure 13. Coomans plot of SIMCA classifications of the European hop varieties Saaz (F) and Spalt (J). Reprinted with permission from Stenroos and Siebert (27). Copyright 1984 American Society of Brewing Chemists.

SIMCA was 100% successful in classifying the samples. However, when looking at pairs of cultivars it was apparent there was some overlap between certain cultivars. The North American variety Clusters was well separated from all the European varieties (see for example, Figure 12). And a number of the European cultivars were easily separated from one another (see for example Figure 13). The Hallertau cultivar, however, overlapped at least two of the other cultivars (see, for example, Figure 14). All of the cultivars but one had at least two significant PCs. The exception was the Hallertau variety, which had no significant PCs. This finding indicates that the samples identified to the procedure as Hallertau were not homogeneous. Some time after this study was concluded an explanation for the results was discovered. The Hallertau variety, while prized as an aroma hop and the most expensive hop cultivar, is quite susceptible to diseases such as mildew. When a hill of Hallertau hops in a field became diseased, in some cases the farmer would remove it and replace it with a hardier cultivar. At this time there were very

few commercial samples of Hallertau hops that were not actually mixtures with other cultivars. SIMCA was able to perceive this.

Multivariate PARC Applications

Pattern recognition has been applied to detecting adulteration or misrepresentation in many different foods and beverages using data from numerous analytical techniques (*4*, *28*). Many of the published applications are in relatively high value foods.

Beverages - Headspace mass spectrometry was used to classify Tempranillo wine by geographic origin using PCA, PLS-DA and stepwise LDA (*29*). German white wines from three districts were classified by NMR and PARC (*30*). Black and green tea based beverages were analyzed for metal composition by inductively coupled plasma atomic emission spectrometry (ICP-AES); PCA, cluster analysis and LDA were then used to discriminate between the beverages (*31*). Various distilled spirits adulterated by addition of water, ethanol or methanol were analyzed by gas chromatography of volatile compounds; the data was then subjected to PCA and SIMCA to very successfully detect the adulteration (*32*). Cachaca (produced by distillation of fermented cane sugar) and rum (produced by distillation of fermented cane molasses) were analyzed for organic compounds, minerals and polyphenols by GC MS, GC FID, HPLC UV and ICAP; the data was analyzed by PCA, hierarchical cluster analysis, discriminant analysis and *k*-NN and produced good discrimination between the two beverage types (*33*).

Honey – Honeys from different geographic areas were adulterated with different amounts of corn syrup and were then analyzed by Fourier transform infrared spectroscopy (FTIR); the data were analyzed by PLS-DA and the adulteration was successfully detected (*34*). Polyfloral and acacia honeys from different geographic origins were analyzed by NMR; PCA distinguished between polyfloral and acacia honeys and between the geographic origins of acacia honeys (*35*). Sugars were determined in honeys from six different floral sources by HP anion-exchange chromatography with pulsed amperometric detection; PCA, LDA and artificial neural networks (ANN) were applied to the data in order to classify the samples (*36*).

Oils and Fats – Compositional data for groundnut, soybean, sunflower seed and corn oils were analyzed by hierarchical cluster analysis (HCA) and SIMCA to classify the edible oils (*37*). Extra virgin olive oils from Mediterranean sources from three crop years were analyzed by NMR; PLS-DA and SIMCA were able to separate Ligurian and non-Ligurian oils (*38*). FTIR analysis was applied to extra virgin olive oil samples from Italy, France, Spain, Greece, Cyprus, and Turkey; PCA, LDA, and PLS were able to successfully classify most of the samples (*39*). Headspace MS analyses of samples of virgin olive oil, olive oil and 'orujo' olive oil were carried out and cluster analysis, PCA, *k*-NN and SIMCA were applied (*40*); SIMCA achieved 97% success in classification. FTIR was applied to coconut oil samples adulterated with cheaper palm oil; PLS-DA was highly successful in detecting the adulteration (*41*).

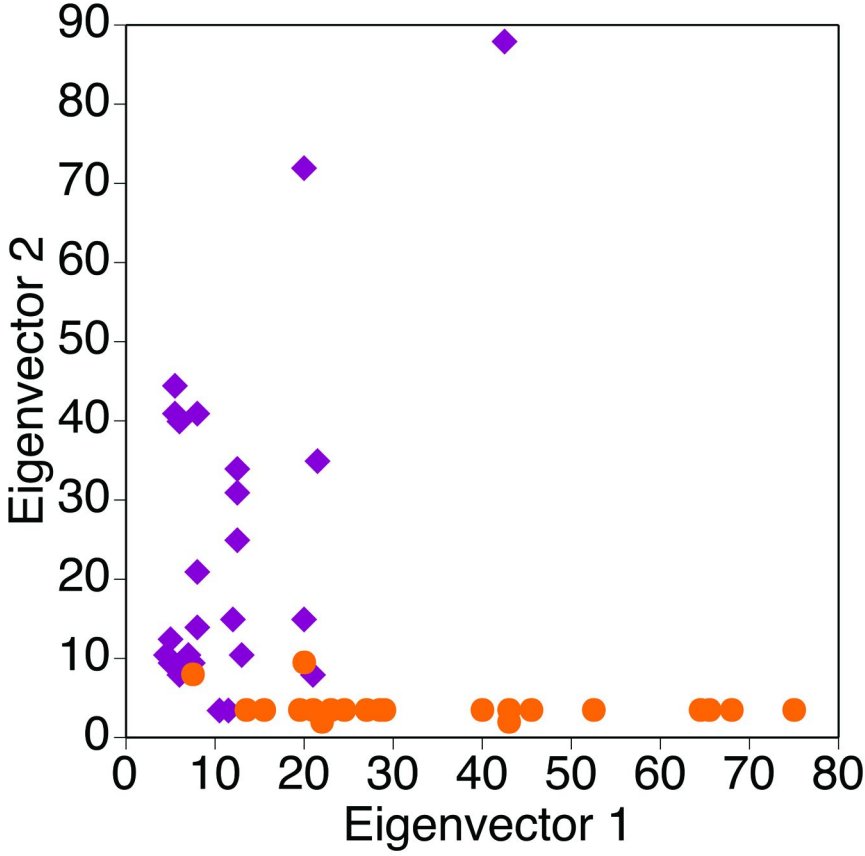

Figure 14. Coomans plot of SIMCA classifications of the European hop varieties Hallertau (F) and Northern Brewer (J). Reprinted with permission from Stenroos and Siebert (27). Copyright 1984 American Society of Brewing Chemists.

Nuts - Hazelnuts from different countries were analyzed for trace elements by ICAP; PCA of the data showed good discrimination by source (*42*).

Dairy/Cheese – NIR and mid-IR (MIR) together with chemometrics have been used to determine cheese quality and authenticity (*43*). A review describes the use of NIR, MIR, fluorescence spectroscopy and NMR applied to various dairy products together with chemometric techniques to evaluate product identity (*44*, *45*).

Fruit –MIR was applied to fruit juices and fractions thereof, followed by HCA and SIMCA; SIMCA was able to differentiate juices by origin (*46*). Authenticity of juice and other products of black currants was studied with chromatography and discriminant analysis (*47*).

Tomatoes - A review of methods for quality control and authenticity determination of tomatoes covers the use of ion chromatography, HPLC, AAS, NMR, sensory analysis and GMO detection methods (PCR and ELISA) together with chemometrics (*48*).

Meat - Various analytical techniques coupled with chemometrics have been used to identify previously frozen meat, identify species of meat, and differentiate wild from aquacultured fish (*49*).

Gums – Carbohydrate gums and mixtures were studied with FTIR and chemometrics; it was possible to detect pure gums and mixtures of gums with PLS (*50*).

General – The use of fluorescence spectroscopy together with various chemometric approaches to characterize food samples (dairy products, eggs, meat, fish, edible oils, peanuts, oatmeal, muesli, honey, wines, and beers) has been reviewed (*51*). Supervised pattern recognition for classification of foods based on chromatographic analysis has also been reviewed (*28*). Detecting adulteration by various analytical approaches (microscopy; HPLC; GC, GC-MS, GC-FTIR; UV-visible spectroscopy; AAS/atomic emission spectrometry (AES), ICP-AES, ICP-MS; IRMS, GC-IRMS, GC-C-IRMS; DSC; IR, MIR and NMR) coupled with chemometrics has been reviewed (*52*). IR spectroscopy together with chemometrics has been applied to fish, dairy powders, wheat flours and coffee beans to detect adulteration (*53*).

Overall

Misrepresentation by either complete or partial substitution of a cheaper ingredient for a more costly one is likely to be the case with an agricultural product. Detecting complete substitution is likely to be simpler than the partial case, particularly where one cultivar is substituted for another. With partial substitution of a solid material, subsamples are likely to vary in composition and in patterns of analytical results. In formulated products like processed foods, adulteration could easily be a matter of degree and naturally a small percent substitution would be both more difficult to detect and less remunerative to the perpetrator of the fraud. In one sense liquids are easier to adulterate because any additive will soon become uniformly distributed, as long as it is soluble in the product. For example, dilution of expensive spirits by water or cheaper spirits in unscrupulous bars is well known.

Which PARC procedure works better than others depends on the nature of the data set and the way the procedure operates. For example, If two classes of samples overlap and are represented by dissimilar numbers of samples in a training set, *k*-NN will assign more unknowns to the class with the larger number of samples than is proper. If some samples belong to a class other than those present in the training set, they will be incorrectly assigned by LDA. SIMCA performs well even when there are few samples compared to the number of measurements.

Multivariate pattern recognition simultaneously examines multiple measurements to seek patterns that characterize samples according to classes. Unsupervised methods simply look at the data set as a whole to see if samples representing various classes naturally tend to group together and to separate from one another. Supervised methods use sample class identifications to derive rules for classification. The training set samples define the class by what they include. Since multiple measurements are used to make classifications and

different PARC procedures emphasize different measurements in a data set to produce classification rules, this can make it difficult and expensive to perpetuate a successful fraud.

Abbreviations

AAS	atomic absorption spectroscopy
AES	atomic emission spectroscopy
ANN	artificial neural network
DA	discriminant analysis
DSC	differential scanning calorimetry
ELISA	enzyme linked immunosorbent assay
FTIR	Fourier transform infrared spectroscopy
GC	gas chromatography
GC FID	gas chromatography with flame ionization detection
GC-FTIR	gas chromatography with FTIR
GC MS	gas chromatography mass spectrometry
GMO	genetically modified organisms
HCA	hierarchical cluster analysis
HPLC	high performance liquid chromatography
ICAP	inductively coupled argon plasma
ICP/AES	inductively coupled plasma/atomic emission spectrometry
IR	infrared spectroscopy
k-NN	*k*-nearest neighbor analysis
LDA	linear discriminant analysis
MIR	mid infrared spectroscopy
NIR	near infrared spectroscopy
NMR	nuclear magnetic resonance
OG	original gravity
PARC	pattern recognition
PC	principal component
PCA	principal components analysis
PLS	partial least squares
PLS-DA	partial least squares discriminant analysis
PRESS	predictive residual error sum of squares
QA	quality assurance
QC	quality control
QDA	quadratic discriminant analysis
RE	real extract
RDA	regularized discriminant analysis
RDF	real degree of fermentation
SIMCA	soft independent modeling of class analogy

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Chapter 5

Authentication Approach of the Chemodiversity of Grape and Wine by FTICR-MS

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The metabolic composition of grapes and related wines results from a complex interplay between environmental, genetic and human factors which are not easily or possibly resolvable into their unambiguous individual contributions. Whether it is in the vineyard or in the cellar, several processes can indeed subtly modulate the characteristics of grape and wine, and these modulations often involve 'trace' amounts and interplay of metabolites within a complex matrix. As a consequence, considering wine as a complex biological system, the whole of which being more than the sum of its parts, is likely to provide deeper understanding of specificities associated with varieties and/or geographical origins and/or wine making practices. We show that non-targeted analyses of grape and wine products by

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ultrahigh resolution mass spectrometry can reveal snapshots of their chemodiversities, and that these chemodiversities, characterized by thousands of metabolites, precisely hold fingerprints of specific environmental parameters such as the "terroir" of a wine or even of oaks used for barrel ageing.

Introduction

Grape or wine authentication relies on the assumption that a physical or a chemical analysis at a given time can provide a "fingerprint" that can relate this wine to a particular variety, region and or a particular process, the three of them being actually joined in many cases. Such a situation can be particularly tricky in Burgundy for instance, where the region of origin of grapes which is often associated with the concept of "terroir" can be as small as an area of the hectare-range size. Physico-chemical approaches have shown great potentialities for the discrimination of wines, as examplified by the "electronic tongue" based on the high cross-selectivity of voltammetric sensors (*1*). However, so far they were unable to provide any detailed structural information on the compounds involved. Similar observations can be made about FTIR spectroscopy, which can be directly applied to wine without any prefractionation steps, and which has also been used successfuly for wine varietal authentication or for wine fingerprinting (*2*, *3*). It is worth noting that when dealing with authentication of wine, methods for stable isotope analysis – in particular site-specific isotope fractionation by deuterium nuclear magnetic resonance (SNIF-NMR) and isotope ratio mass spectrometry (IRMS) – assume a particular importance owing to their official nature for the detection of adulterations (*4*). However, if such methods have also been used efficiently for the discrimination of geographical origins of wines, they basically rely on the analysis of the two major components of wine: water and ethanol. Analyses of various wine chemical constituents on the other hand have been thouroughly investigated in the last decades and considerable progress has been made in the characterisation of grape and wine metabolites in general (*5*). Beyond basic chemotaxonomic approaches, both therapeutic and organoleptic issues have indeed fueled numerous analytical studies because of the crucial role played by grapevine and wine macromolecules and secondary metabolites on the flavour, stability, therapeutic activity for moderate consumption and consequently on the wine industry (*6–11*). However, the vast majority of such wine analyses up to date have relied on a molecular targeted basis, *i.e.* they were based on the selective analysis of families of compounds through the implementation of various chromatographic-based approaches (*12*). They have assumed and often confirmed the presence of molecules in wine, in correlation to the particular property under investigation (organoleptic, therapeutic...). Targeted analyses of selected compounds that could provide fingerprints of wines have also been reported (*13*, *14*), but the most promising approaches appear to be based on the implementation of non-targeted analyses of grapes and wines associated with multivariate data analysis. In that respect, Nuclear Magnetic Resonance and high-resolution Mass spectrometry are undoubtly the most efficient tools because they can be

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directly applied to wines with minmum sample preparation (*15–20*). These two methods are however characterized by distinct advantages and drawbacks: the major limitation of NMR is its low sensitivity and so far discriminations were only based on limited numbers of compounds, but its versatility can allow the structural identification of some of these compounds. In contrast, ultra high resolution mass spectrometry allows one to identify large numbers of discriminant elemental formulas but without the possibility to unambiguously determine the contributions of the different isomers to these particular formulas, unless it is hyphenated with additional MS tools. High resolution mass spectrometry can provide though a highly resolved instantaneous picture of a complex biological system and thus reveal how diverse its chemical composition can be (*15*).

It is indeed generally accepted that flavours and chemical compositions of wines are not only related to genetic factors (grape variety) but also to environmental conditions in vineyards (climate, soil composition and geology, microflora) and to human practises. Metabolic changes occur throughout the growth and maturation of grape berries, and at harvest time the berries contain the major grapevine compounds contributing to the wine organoleptic characteristics (*21*). During winemaking processes and in particular during fermentation, these compounds act as sources of carbon, nitrogen and other elements for yeasts, and are either further metabolised, chemically transformed or directly transferred to the wine. Yeast metabolism will further contribute to the wine enrichment through, for instance, the enzymatic liberation of particular volatile organic molecules responsible for the aroma of wine. Therefore, even if the biochemical and functional-genomics approaches of enzyme signaling are successful in determining how the accumulation of active compounds is regulated at the molecular level in the grapevine or the grape (*22–24*), they are not sufficient for providing an integrated picture of the subtle process-related synergistic modulations of metabolites within a complex matrix that is ultimately considered as a unique beverage (*25*).

Here, we will show that the non-targeted metabolite analysis of a series of wine samples from the "Tonnellerie 2000" experiment (*26*), by ultra high resolution Fourier Transfrom Ion Cyclotron Resonance – Mass Spectrometry (FTICR-MS), can reveal the extremely high, yet unknown diversity of metabolites involved in the different steps of the elaboration of wines. Furthermore, we show that such diversity in wines can hold complex fingerprints of grape varieties, regions of origin, species and even geographic origins of oaks used for barrel ageing of wines.

Materials and Methods

Barrels Elaboration

The "Tonnellerie 2000" experiment has been designed to particularly take into account the high interindividual variability which had already been observed even between trees from the same forest. Therefore, the selected procedure was based on the combination of many trees considered as representative of one species in one forest.

The detailed procedure followed to select trees has already been described elsewhere (*26*). In brief, twelve lots (5 pedunculate and 7 sessile) of 24 trees were selected from nine French forests.

To one lot of 24 trees corresponded one barrel. Each barrel has thus been assembled from 24 trees which stood each for 1/24th of the toasted surface (body) and 1/24th of the untoasted surface (head and bottom). After 13 months of natural drying some of the staves were selected. Forty-eight barrels (12 lots x 4 repeats) were assembled and subsequently medium toasted for 45 minutes. These barrels were used for wine ageing experiments with the red Pinot noir wine from Mercurey and the white Chardonnay wine from Beaune. The remaining staves were further dried for an additional 43 months (giving a total drying duration of 56 months). Forty-eight new barrels were then assembled following the same procedure as before for wine ageing experiments with a red Syrah wine from Côte Rôtie (Côte du Rhône north) and a red Grenache wine from Gigondas (Côte du Rhône south).

Wines Elaboration

The first set of two experiments was designed during the 1998 harvest; one with the appellation "Mercurey rouge 1er cru" with "Pinot noir" variety (12 lots x 2 replications + 1 reference stainless steel tank), and the other with the appellation "Beaune 1er cru" with "Chardonnay" variety (12 lots x 2 replications + 1 reference stainless steel tank). At the end of the wine ageing period (11 months for the red, and 13 months for the white), wines were bottled after blending of the two replicates for each lot, thus providing 13 bottles of Mercurey and 13 bottles of Beaune. The second set of two experiments was similarly designed during the 2002 harvest, one with the appellation "Côte Rôtie" with "Syrah" variety (12 lots x 2 replications + 1 reference stainless steel tank), and the other with the appellation "Gigondas" with "Grenache" variety (12 lots x 2 replications + 1 reference stainless steel tank). For the Côte Rôtie wine, wines were bottled after 12 months of oak ageing but separately for the two replicates. Therefore, we had at our disposal 2 sets of 12 bottles plus the bottle of wine from the stainless steel tank. For the Gigondas wine, bottling has completed after 6 months of oak ageing and after blending of the two replicates.

Wine Sample Preparation

Wines from the Tonnellerie 2000 experiment were sampled directly from the bottles through the cork using a Hamilton needle. Only 20 µL of wine was diluted into 1 mL methanol from which only 50 µl was used for one experiment and to reach the spectral quality presented herein.

Pinot Noir Wine, Must, and Skin Extract

100 Pinot noir berries were collected at harvest. The must and skins were separated by pressing the berries in a laboratory-scale press, and skins were subsequently dried on filter papers. Skin extracts were obtained by crushing (Ultra Turrax, IKA, Wilmington) skins in pure methanol (LC-MS grade) and the

procedure repeated twice. Mixtures were then centrifuged (10 mn, 25400 *g*) and 20 µl of supernantant were diluted in 1 ml of methanol prior to injection. The must sample was obtained by separation on a C18 cartridge and an elution with methanol. 20 µl of the eluted solution were diluted in 1 ml of methanol prior to injection. The corresponding wine was collected immediately after the alcoholic fermentation.

Yeast Fermentation Medium

A synthetic fermentation medium with *Saccharomyces cerevisiae* cells was prepared with the following composition for 1 liter and pH 3.5: 120 g glucose, 120 g fructose, 2 g tartric acid, 10 g D,L malic acid, 0.5 g NH_4Cl, 0.6 g Yeast Carbon Base and water. When the total sugar concentration reached a concentration lower than 2 g/l, indicating the end of the fermentation process, the hydroalcoholic medium was centrifuged (5 minutes, 10000 g) and the supernatant recovered. 20 µl of this supernantant were diluted in 1 ml of methanol prior to injection.

Oak Wood Extracts

Methanolic extracts were obtained by soaking approximately 500 mg of untoasted oak wood shavings (blend of sessile and pedunculate species) in 5 ml of methanol for 30 minutes in an ultrasonic bath. After centrifugation (5 minutes, 25400 g) 20 µl of the supernantant were diluted in 1 ml of methanol prior to injection.

FTICR-MS Analysis

High-resolution mass spectra for molecular formula assignment were acquired on a Bruker (Bremen, Germany) APEX Qe Ion Cyclotron Resonance-Fourier Transform Mass Spectrometer (FTICR-MS) equipped with a 12 Tesla superconducting magnet and a APOLO II ESI source in the negative ionisation mode. Samples were introduced into the microelectrospray source at a flow rate of 120 µl/h with a nebuliser gas pressure of 20 psi and a drying gas pressure of 15 psi (200 °C). Spectra were externally calibrated on clusters of arginine (10mg/l in methanol) and accuracy reached values lower than 0.1 ppm in day-to-day measurements. Further internal calibration was done for each sample using fatty acids and accuracy reached values lower than 0.05 ppm. The spectra were acquired with a time domain of 1 megaword (4 megaword for selected samples) with a mass range of 100–2000 m/z. The spectra were zero filled to a processing size of 2 megawords and an average resolution of 250.000 was reached at m/z 200 (100.000 at respectively m/z 600) in full scan. Before Fourier transformation of the time-domain transient, a sine apodization was performed. The ion accumulation time in the ion source was set to 0.2 s for each scan. 1024 scans were accumulated for samples.

Statistical Analyses

Raw data (mass spectra) were normalised, and then transformed to log(X + 0.00001). The constant 0.00001 was added to provide non-detectable components with a small non zero value (*27*). Transformed variables were then mean centered and Pareto scaled and represented as an X matrix. Pareto scaling gives each variable a variance equal to its standard deviation by dividing by the square root of the standard deviation of each column (*28*). The sample classification and the prior information about the sample were done using the Hierarchical clustering analysis (HCA) unsupervised method. On the other hand, partial least square – discriminative analysis (PLS-DA), performed with SIMCA 11.5, was used to discover characteristic biomarkers (*29*). This multivariate procedure provided bioinformatics clues for the selection of a limited number of masses most effective in discriminating different species and forests.

The feature selection procedure comprises two steps: i) identification of those masses that best describe each class (a list based on the modelling power of the original variables), and ii) scoring and ranking of the variables in every class-related list according to their abilities to discriminate the class they model from all other categories. The ranking and scoring take place after computation of the minimum number of masses through the formula generator (in-house code written in FORTRAN). The generated formulas were validated by setting sensible chemical constraints (N rule, O/C ratio ≤ 1, H/C ratio $\leq 2n + 2$, element counts: $C \leq 100$, $O \leq 80$, $N \leq 5$, $S \leq 1$) and only the masses in conjunction with their generated theoretical isotope patterns were taken into consideration.

Results and Discussion

The Chemical Spaces of Wine

Chemical spaces of wine can be as diverse as there are steps involved in the elaboration of a wine, and even within each of these steps, a chemical space will be subject to various environmental influences. We can characterize such a chemical space by the number of elemental formulas that can be identified in the mass spectrum of the ionised metabolites under the selected experimental electrospray conditions (*30*). As an example, Figure 1 shows the negative mode mass spectra of methanol dilutions of must and skins of Pinot noir grapes. Such overall spectra, which can exhibit several thousands of resolved peaks within the detected 150-2000 m/z range, already display different peak patterns which highlight the distinct chemical spaces that will contribute to the initial wine chemistry before the onset of yeasts fermentation.

These spectra are actually characterized by a diversity of chemical spaces that can be better observed in the mass distributions within the 300 millimass range of the 477 nominal mass of the spectrum of a grape skin extract (Figure 2). Up to 13 distinct signals can be indeed seen, of which eight signals can be assigned an unique CHO absolute formula (Table 1). Two homologous series can actually be seen with identical mass defect intervals corresponding to a formal compositional

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exchange of O for CH_4 (mass difference of 36.4 mDa). However, a particular feature of these series, which is regularly observed throughout the entire spectrum, is the non continuous intensity distribution within a nominal mass. In Figure 2 (and Table 1) for instance, a first series (peaks 1 and 2) only displays peaks at low mass defects (high oxygen contents) whereas the second series (peaks 3 to 8) rather extends to high mass defects (higher hydrogen contents) and with a non-gaussian shape. Such non-gaussian intensity distributions are characteristics of the biological origin of the mechanisms of formation of the associated compounds where the highest probability in the number of feasible isomers (for a given absolute formula) is not centered at intermediate mass defects (*31*, *32*). Interestingly, only two of these peaks can be associated with known chemical structures pertinent to the grapes chemical composition, thus emphasizing a yet unknown chemical complexity. The peak at m/z 477.0674 (peak 1), which corresponds to the $[M-H]^-$ ion with absolute mass formula $[C_{21}H_{17}O_{13}]^-$ could possibly be associated with the quercetin glucoronide bioflavonoid whereas its homologous peak at m/z 477.1038 (peak 2), which corresponds to the $[M-H]^-$ ion with absolute mass formula $[C_{22}H_{21}O_{12}]^-$ could possibly be associated with the isorhamnetin glucoside (*33*).

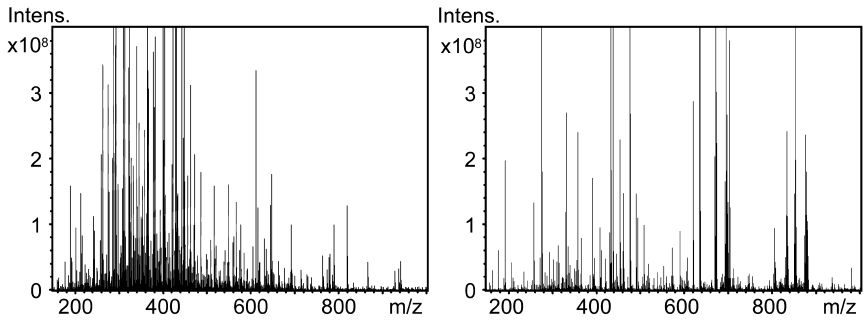

Figure 1. Negative ion FT ICR mass spectra of a Pinot noir must (left) and of a Pinot noir skin extract (right) on the 150 – 1000 m/z range.

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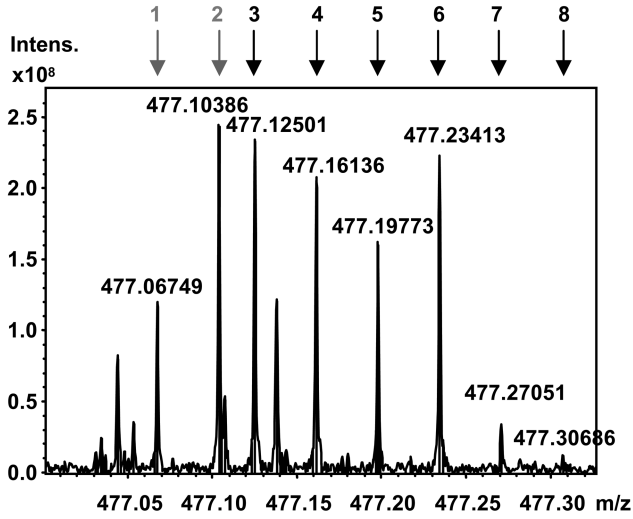
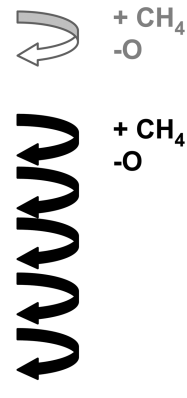


Figure 2. Detail on the nominal mass 477 (FTICR-MS spectra in negative mode of a Pinot noir skin extract) showing up to 13 disinct signals within a 300 millimass range, of which up to eight signals can be assigned an unique CHO absolute formula (see Table 1); two homologous series of signals (grey and black arrows) can be observed which vary by a formal exchange of O against CH_4 (Table 1).

Table 1. CHO absolute formulas attributed to the two homologous series of signals observed in Figure 2

Signal	Formula
1	$C_{21}H_{18}O_{13}$
2	$C_{22}H_{22}O_{12}$
3	$C_{19}H_{26}O_{14}$
4	$C_{20}H_{30}O_{13}$
5	$C_{21}H_{34}O_{12}$
6	$C_{22}H_{38}O_{11}$
7	$C_{23}H_{42}O_{10}$
8	$C_{24}H_{46}O_9$



van Krevelen Diagrams

Clearly, the handling of such high dimensional non annotated mass peaks for any given spectrum necessarily requires advanced chemometric approaches. However, an initial interpretation of such compilations of masses is made with two-dimensional van Krevelen diagrams (*34*, *35*), which sort each elemental composition onto two axes according to its H/C and O/C atomic ratios. Unprecedented graphical representations of the various chemical spaces of wine are then obtained (Figure 3). van Krevelen diagrams allow the sorting of elemental compositions into chemical families and therefore provide a visual qualitative description of chemical spaces which transiently contribute to the final chemistry of a wine (*15*). Indeed, when analysed separately, each step of the elaboration of a wine can be characterised by the potential release into the wine of thousands of compounds of extensive molecular diversity (Figure 3).

In the CHO compositional space, a grape must sample would bring a wide diversity of compounds, from saturated oxygen depleted to unsaturated and highly oxygenated compounds, though with a predominance of rather saturated compounds (H/C ratios > 1.5) in the regions of fatty acids, amino acids and sugars. In contrast, a skin extract would be dominated by tannins and anthocyanins polyphenolic structures, though many other compounds with higher H/C ratios could also be extracted. In a chemical space restricted to CHO molecules, a *Saccharomyces cerevisiae* culture medium would appear poorer, though still containing hundreds of distinct molecules. However, expanding the chemical space to CHONS elemental compositions, as illustrated for the *Saccharomyces cerevisiae* culture medium (Figure 3), reveals a much larger chemical diversity of nitrogen and/or sulfur containing metabolites typical of the yeast core metabolome (*36*). It must be borne in mind that, FTICR-MS alone does not allow one to distinguish isomers, therefore, it is likely that in any of the observed chemical spaces, the actual chemical diversity is considerably higher than that derived from mass peaks alone (*31*). During the elaboration of wines, barrel aging is an important environmental factor. Indeed, initially aimed at being suitable containers, oak barrels became a practical means of modulation of fine sensory characteristics of wine. Several studies have been devoted to the modifications undergone by the wine during oak barrel aging, with particular emphasis on the aromatic complexity provided by the contact with more or less toasted wood staves, in conjunction with low oxydation conditions enabled by this porous container (*37*). Barrel aging is a striking example of the extremely complex modifications that a wine can undergo (*38*). In addition to natural clarification, colour stabilisation is favoured by ellagitanins extracted from oak wood and other acid catalysed reactions between hydrolysable tannins and wine nucleophiles (*39*), and oak wood can also act as a sorbent, with an appreciable selectivity towards hydrophobic metabolites of wine (*40*). Thousands of molecules can actually be extracted from oak wood barrels, with a clear distinction of their nature according to the level of toasting (*15*). As an example, an untoasted wood extract is characterized by a huge amount of rather oxygenated compounds in the CHO space, which are mostly found in the polyphenolic region of the diagram (Figure 3), whereas at increased temperatures of toasting, more extensively oxygen

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depleted derivatives are formed. Finally and remarkably, the resulting wine will be characterized by an overall chemical space which is definitely more than the sum of its transient contributions because of the molecular diagenesis allowed in the bottle during its ageing (*15*). As can also be seen in Figure 3, relative proportions of CHO, CHOS, CHON, CHONS subspaces clearly vary from one step to the other in the elaboration of the wine. Under similar experimental conditions, the must, the skin extract and the wood extract would be characterized by a major proportion of CHO compounds whereas the yeast culture medium would be dominating by CHON and CHONS compounds. Interestingly, we can observe in Figure 3 that the distribution of subspaces for a red wine that has just finished its alcoholic fermentation – therefore where an in-bottle molecular diagenesis has not started – actually shows some similarity with the distribution of subspaces of its corresponding skin extract. Although these results already reveal an unprecedented diversity of metabolites which can be potentially present in wine, it must be born in mind that the presented chemical spaces do not provide an exhaustive chemical picture of a biological system because not all of the present metabolites can actually ionise under the selected experimental conditions (acidic-like compounds will more easily ionise in negative mode (*30*)) and furthermore, only a rather narrow mass range is explored. For instance, many of the compounds responsible for the aroma of wines, which exhibit m/z values below 150, were not detected under our experimental conditions.

Wine Fingerprints of the Grape Variety

Beyond the basic metabolomic approach of the description of wine chemical spaces, FTICR-MS can also be used as an holistic non-targeted approach, which enables an instant molecular picture of a complex biological system. As such, it allows a non-targeted metabonomics approach (*41*), which combines multivariate statistics with high-dimensional unannotated variables, and offers the possibility to integrate all the history of time-related metabolic changes of wine throughout its elaboration process. In this context, it could be assumed that chemical spaces of a wine may hold both genetic and chemical information on environmental influences that operated during its elaboration. Therefore, even after bottling these chemical spaces of wine should still hold fingerprints related to the grape variety they were elaborated from or to the vineyard ecosystem.

In 1998, a full-scale integrated study involving 9 French forests and 4 sets of French wines was designed to evaluate the influence of the geographic origin and the species of oak wood on the quality of wines matured in oak barrels (*26*). Each of these 4 sets corresponded to 12 repetitions of the same wine, which only differed by the oak wood species and origin of the trees used for the elaboration of barrels they were aged in.

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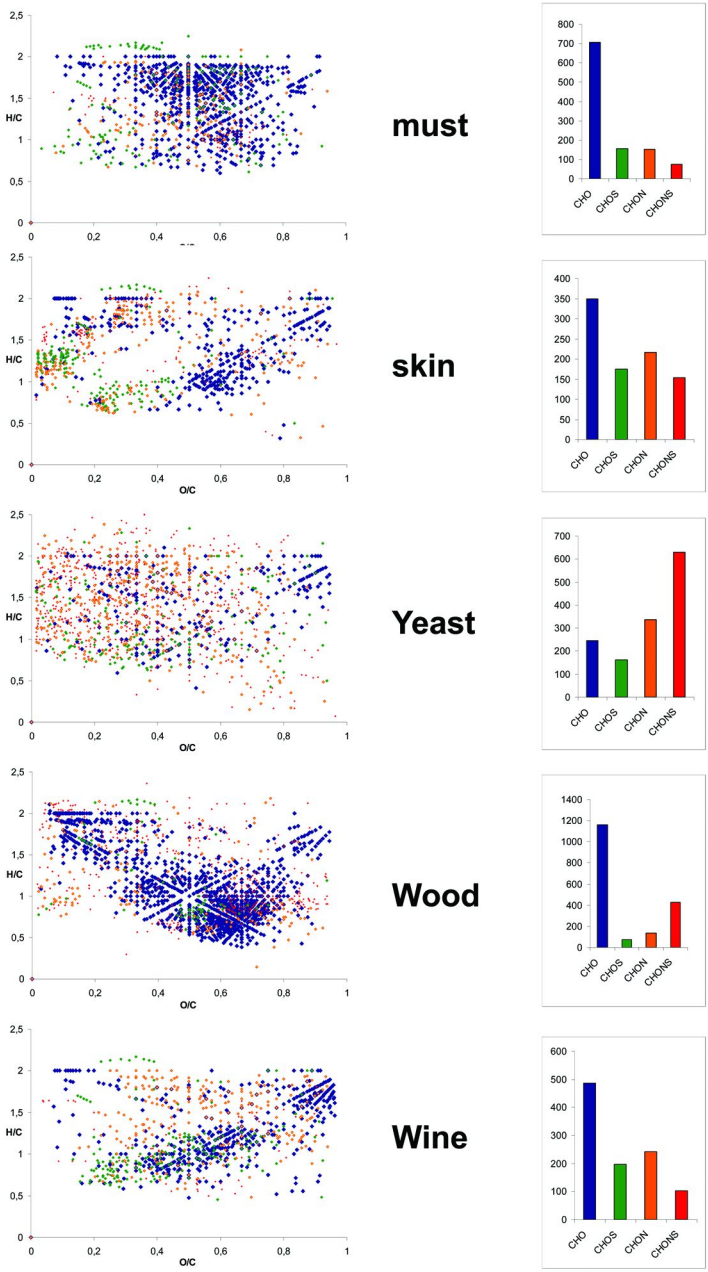

Figure 3. van Krevelen diagrams (left) and corresponding distributions of elemental compositions (right) representing the various chemical spaces which transiently contribute to the chemodiversity of a wine. (see color insert)

We recorded the negative and positive-ion electrospray ionisation mode FTICR-MS mass spectra of each of these 60 wines and these data were further statistically processed in order to identify possible discriminations among wines (only the negative ion electrospray data are shown here / positive ion data showed the same differentiations). PLS-DA score plots of wines according to their geographical origin illustrate the very good discrimination allowed by FTICR-MS, with an average of about 300 distinct and annotated discriminant compounds for each region (Figure 4). Although analytical discriminations of wines based on the regions of origin have already been largely reported (*17*), these results clearly appear to complement them in terms of the unprecedented chemical diversity that is responsible for the discriminations. Figure 4 indeed shows for instance, that among the two southern regions, signals specific to Gigondas wines (made of Grenache grapes) span from the lower left corner of the van Krevelen diagram (O/C about 0.2 and H/C about 0.6) up to the upper right corner, whereas Côte Rôtie wines (made of Syrah grapes) are mostly discriminated by compounds in the polyphenolic region. Although our analyses were initially not intended for regional discrimination, these results interestingly suggest that some of the parameters (grape variety, terroir, elaboration practices) that could have had an impact on the initial chemical composition of the wine (before bottling) for a given region can potentially be traced out in the aged wine even after several years in bottle. Indeed, the observation for instance of more oxygenated compounds in Gigondas wines would be consistent with the oxydative process frequently used for their elaboration. However, more replicates would be necessary to unambiguously confirm these results.

As already mentioned, the major limitation of ultra high resolution FTICR-MS is that it only provides precise elemental formulas but does not give the associated molecular structures. Therefore, if hundreds of distinct elemental formulas can be detected here, unambiguous structural identifications of discriminant compounds cannot be done at that point. However, reliable structural assumptions can be drawn from the questioning of topic related available databases such as KEGG, accessible with the MassTRIX interface (*36*), and the discrimination of wines according to grape varieties (regardless of any other parameters) is an excellent assessment of the *Vitis Vinefera* pathways accessible through this interface. Hence, when all the exact masses that discriminate the four wines are processed through MassTRIX, between 20 and 29 distinct formulas are found for each wine from the different annotated *Vitis vinifera* organism pathways (Figure 5). Most interestingly, these results indicate that only a few hits are found among the different polyphenolic-related pathways to discriminate the three red wines (Mercurey, Côte Rôtie and Gigondas). This clearly points to the molecular diagenesis that occurs upon ageing in bottles, since for all of the four wines less than 10% of the overall discriminant masses can actually find hits in the different *Vitis vinifera* pathways, thus showing the importance of the yet structurally unresolved chemistry of wine. As examples among the possible metabolites identified in *Vitis vinifera* pathways, the peak at m/*z* 149.04554, to which can be unambiguously assigned the $[M-H]^-$ ion with absolute mass formula $[C_5H_9O_5]^-$, possibly corresponding to pentose sugars such as lyxose, xylose or arabinose, would discriminate the southern Gigondas samples. Mercurey and Côte-Rôtie

wines would be both discriminated by N and S-containing molecules such as for instance the cystathionine amino acid for the former (m/z 221.06004; absolute ion mass formula $[C_7H_{13}N_2O_4S]^-$) and the antioxidant gluthatione tripeptide for the latter (m/z 306.07651; absolute ion mass formula $[C_{10}H_{16}N_3O_6S]^-$). Interestingly for the Côte Rôtie wines, the peak at m/*z* 321.06116, to which can be unambiguously assigned the $[M-H]^-$ ion with absolute mass formula $[C_{15}H_{13}O_8]^-$, could correspond to the trihydroxylated leucodelphinidin monomer (*42*) which, to the best of our knowledge has never been found in aged wines so far.

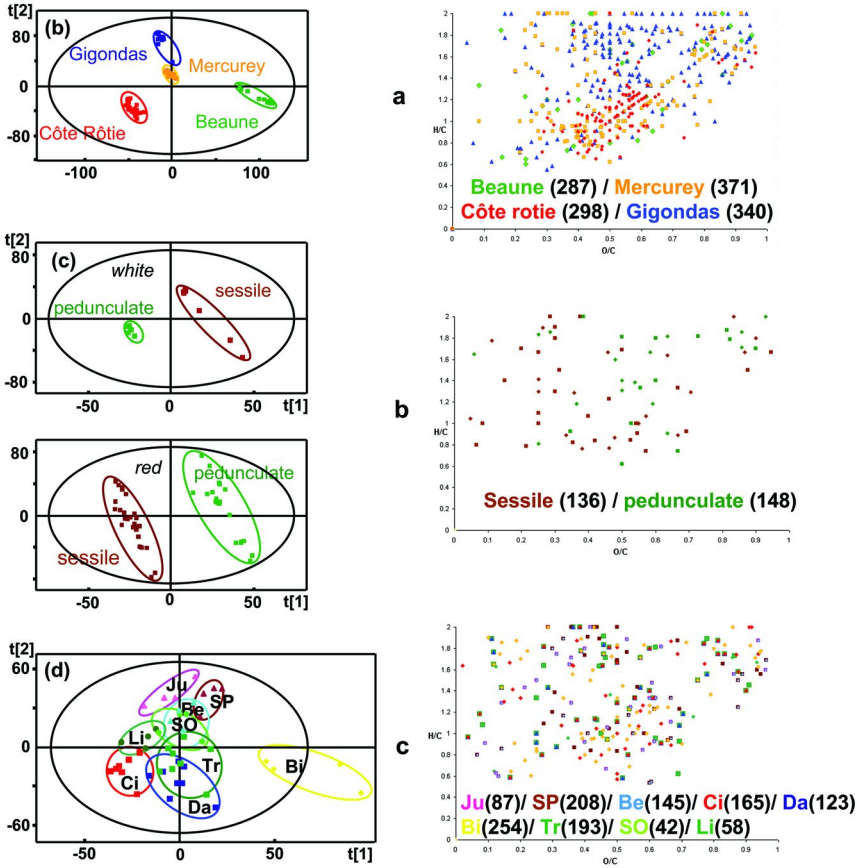

Figure 4. PLS-DA score plots (left) with van Krevelen representation of the associated discriminant elemental formulas (right) of wines from the "Tonnellerie 2000" experiment (adapted from ref. (15)); In brackets: number of distinct dicriminant elemental formulas for each class . Colour codes are (a) Gigondas (■), Mercurey (■), Beaune (■), and Côte Rôtie (■) wines; (b) White and red wines aged in Sessile (■) and Pedunculate (■) barrels; (c) wines sorted according to forests of origin of oaks of barrels they were aged in, regardless of the species: (Ju) Jupilles, (SP) Saint Palais, (Be) Bertrange, (Li) Limousin, (SO) Sud Ouest, (Tr) Tronçais, (Ci) Citeaux, (Da) Darney, (Bi). (see color insert)

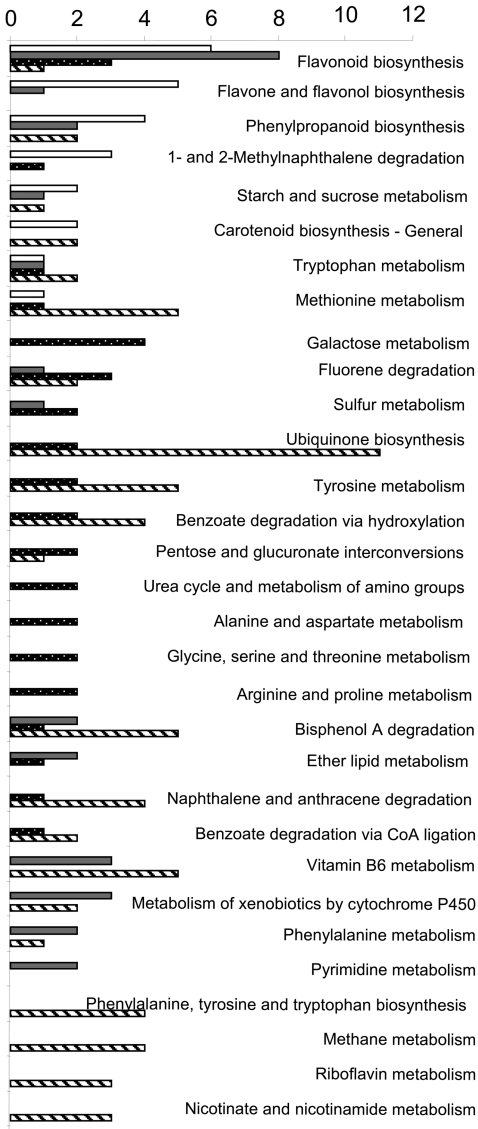

Figure 5. Histogram plots of the number (N) of metabolites discriminating the regions of origin of wines of the Tonnellerie 2000 experiment, which can be found in pathways of the Vitis vinifera organism, as annotated from ICR-FT/MS data and using the Masstrix translator into pathways interface: Mercurey (▬), Beaune (▭), Côte Rôtie (▬) and Gigondas (▬),

Wine Fingerprints of Oaks Species

The discrimination of wines according to the oak species of the barrels they were aged in, on the basis of significantly higher amounts of aromatic whiskylactones in wines aged in European sessile or American white oak barrels (*43*), has been realized for a long time. However, our PLS-DA score plots for both red and white wines, along with the van Krevelen representation of the corresponding discriminant formulas, provide an enhanced representation of how wines aged in barrels from a given wood species are grouped together (Figure 4). We can observe a significantly narrower distribution among white wines aged in pedunculate barrels than among those aged in sessile barrels, whereas no such difference in distribution is observed for red wines. For white wines, these findings corroborate the previously observed narrower distribution among pedunculate oak wood extracts than among sessile oak extracts (*44*). In contrast, the broader distribution among red wines aged in pedunculate barrels witnesses to the multiple – yet to be discovered – products of the possible reactions between ellagitannins and wine nucleophiles such as polyphenolic compounds characteristic of red wines (*39*). However, under our experimental conditions, none of the known flavanol-ellagitannin condensation products (*39*) were detected. As also shown by the van Krevelen diagram, it is worth of note that discriminant compounds for sessile related wines are on average more oxygen depleted than pedunculate related wines.

Wine Fingerprints of Oaks Origin

Figure 4 finally shows that for the first time, analytical-based experiments were able to discriminate wines according to the origin of the forest of the oaks used for barrel aging of these wines. As expected though, the discrimination is better obtained when forests of only one given species are considered, and particularly for the pedunculate species, which is all the more clear with the three forests having both species (Citeaux, Tronçais, Darney) (Figure 6). This interesting result might be (partly) explained by the fact that sessile oaks are chemically characterized by more aromatic and volatile compounds than pedunculate oaks. Therefore, whether diagenesis has occured or not, many likely candidates for the discrimination of sessile oak forests would have low masses and would consequently not be detected under our experimental conditions. In contrast, the chemistry specific to each pedunculate oak forest would be more accessible to our FTICR-MS set up because of the higher masses of the compounds involved. In Figure 4, the comprehensive two-dimensional van Krevelen diagram provides a representation of chemical spaces that discriminate forests, regardless of the colour, the origin of production (and grape variety), and the barrel oak species. In particular, it clearly shows that compounds responsible for the discrimination not only include polyphenolic-related species but also span from saturated weakly oxygenated molecules to unsaturated highly oxygenated ones. It must be remembered that such discrimination is necessarily based either on wood extractables or on related products of the molecular diagenesis that could have occured upon ageing. An interesting and surprising example

of possible discriminant metabolite is taken from the more than 250 elemental formulas specific to wines aged in oaks from the Bitche forest, which as shown in Figures 4 and 6, are significantly discriminated from others. In this forest, considerd to be an unique sessile oak ecotype (*45*), with extreme conditions for the production of cooperage wood, oaks are mixed with the frugal scots pines and grow on dry acidic soils resulting from the decompostion of sandstones from the Vosges. These conditions explain the slow growth of these oaks and the corresponding low values for their physical parameters (*46*), which are in turn likely correlated to some of the various metabolites that discriminate this forest in wines. Another remarkable characteristic of this forest is the presence of an exceptional biodiversity of epiphytic lichens, some of them being very rare (*47*), which is a marker of high ecological quality of the forest. Now, the ionic formula $[C_{19}H_{17}O_8]^-$ which can be unambiguously assigned to the signal at m/z 373.0929 only observed (with S/N > 2) in wines aged in Bitche barrels, finds only one hit relevant to aged wines in SciFinder Scholar: the hydroxy-tetramethoxy flavan-dione oxydation product of quercetin, observed in model conditions (*48*). However, if an oxydation product of quercetin is certainly consistent with the chemistry of aged wines, there is no explanation why it is only detected in Bitche related wines. A second remarkable hit can actually be found in the literature: the known bitter Atranorin lichen product, supposed to be transferable from the lichen to its oak carrier (*49*). Although such assumption seems to be rather surprising, it appears to be consistent with the particular ecology of the Bitche forest. In such case, a lichen metabolite would be a marker of cooperage oaks from the Bitche forest in wines. It must be noted though, that possible off-flavour consequences associated with this bitterness have not been detected since preliminary sensory analyses showed a preference for wines (Côte-Rôtie) aged in Bitche barrels (*50*).

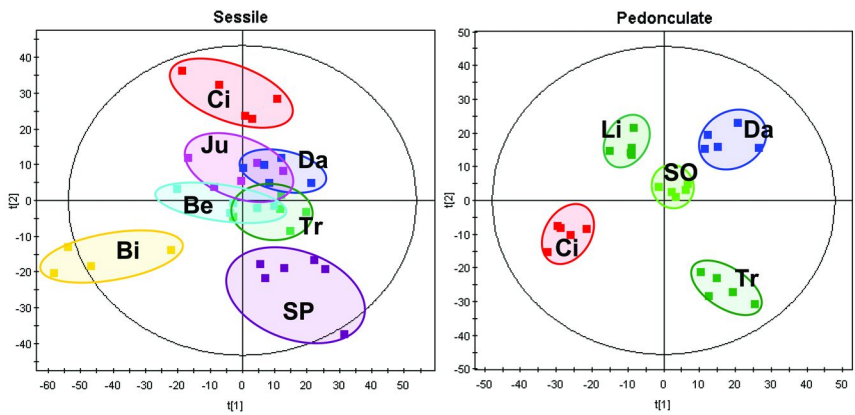

Figure 6. PLS-DA score plots of wines from the "Tonnellerie 2000" experiment, sorted according to forests of origin of oaks of barrels they were aged in, for sessile oaks (left) and pedunculate oaks (right); (Ju) Jupilles, (SP) Saint Palais, (Be) Bertrange, (Li) Limousin, (SO) Sud Ouest, (Tr) Tronçais, (Ci) Citeaux, (Da) Darney, (Bi). (see color insert)

Conclusion

Throughout these results, we have shown that approaches aiming at the most comprehensive representations of wine through its diverse chemical spaces, considerably enhance the opportunities of discriminating metabolites related to distinct environmental conditions with likely outcomes regarding their impacts on organoleptic or therapeutic activities. Through the unique experimental set up of the "Tonnellerie 2000" experiment, this study revealed that even after several years in bottles, wines could still hold distinct chemical fingerprints of their regions of origin (regardless of the barrel oak species and origins), of the oak species of barrels they were aged in (regardless of their regions of origin, and of oak origins) and finally of the geographical origin of the oak barrels they were aged in (regardless of their regions of origin, and to some extent of oak species). These analytical results are not only remarkable in that they reveal that a wine can hold a memory of environmental parameters related to its elaboration, but also in that this memory is actually characterized by an unprecedented diversity of metabolites, as illustrated by the three-dimensional van Krevelen representation (Figure 7) of the cumulated 3492 discriminant signals, to which unique CHO-based chemical formulae could be assigned. We can see that within the 150-1000 m/z mass range, discriminant compounds cover a wide range of chemical structures and span from rather saturated oxygen-containing compounds at low masses to rather condensed oxygen-depleted compounds at higher masses. In the particular case of barrel ageing of wines, our *systems oenology* approach provides an unprecedented example of metabologeography (*51*) in wine, where the concept of terroir of oaks is extended to forests regardless of the oak species.

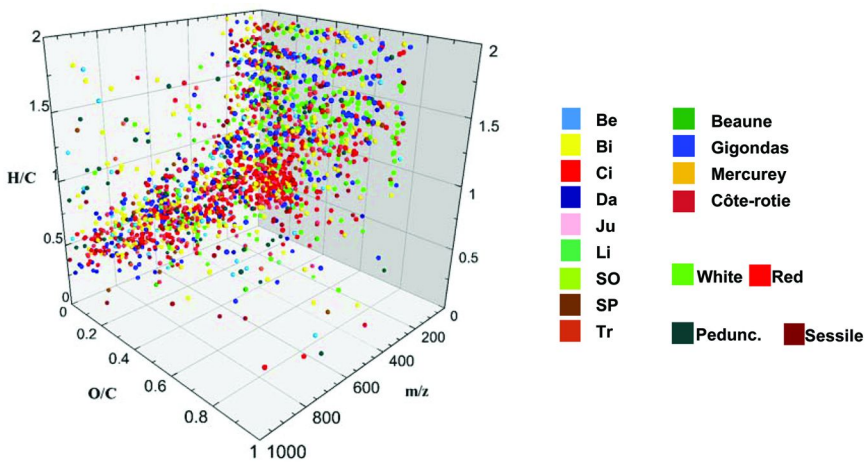

Figure 7. Three-dimensional van Krevelen diagram of the cumulated 3492 discriminant signals of all of the discriminated classes within the Tonnellerie 2000 wines, to which unique CHO-based chemical formulae could be assigned. The third dimension is the m/z mass. (see color insert)

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Chapter 6

Authentication of Red Wine Vintage Using Bomb-Pulse ^{14}C

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As a result of atmospheric atomic explosions carried out in the 1950's to 1963 a potential method for the short term dating of wines has emerged based on the determination of ^{14}C in wine components. The atmospheric nuclear explosions released relatively large quantities of ^{14}C into the atmosphere which was incorporated into carbon dioxide. The immediate post test $^{14}CO_2$ concentration in the atmosphere initially decreased due to its uptake in marine and terrestrial carbon reservoirs. More recently, the dilution of $^{14}CO_2$ in the atmosphere has been driven by the burning of fossil fuels which are depleted in ^{14}C (because of radioactive decay).The uptake and sequestering of atmospheric CO_2 by plants means that by accurately measuring the amount of residual ^{14}C in plant derived-products, the year when that sequestration took place can be determined. The amount of ^{14}C in grape-derived components in the wine, such as alcohol, has been used to reliably determine red wine vintage. Measurement of the residual levels of ^{14}C in additional components in the wine, such as the phenolic compounds, makes the method more robust and less easily circumvented by illicit additions.

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The authentication of Australian wine vintages and the detection of unauthorized additives and wine 'forgeries' are of major importance to the wine industry. Various methods have been used to distinguish the addition of materials of natural and synthetic origin to foods and wines. These methods include the use of stable isotope ratios measured by isotope ratio mass spectrometry (IRMS) such as $\delta\ ^{18}O/^{16}O$ and $\delta\ ^{13}C/^{12}C$ to investigate water and alcohol additions to juices and wines (*1*). Additionally, measurement of $\delta\ ^{2}H/^{1}H$ ratios at specific sites within target molecules by NMR (SNIF-NMR) has been used to detect, for example, the addition of sugar from cane or beet in fermentations (*2*). Whilst the methods provide a measure of authentication of origin, and they can also be used to identify unauthorized additions and adulteration; but the determination of the age of the product, and in the case of wine, its vintage is less easily achieved.

A technique that is aimed at determining wine vintage targets the age of the bottle based on the elemental composition of glass. The bottle is bombarded with a high energy proton beam and differences in glass composition gives rise to characteristic X-ray emissions (PIXE) to give a signature or fingerprint which can be compared with authentic standards (*3*). The method is potentially open to manipulation in that the bottle does not necessarily reflect its contents but by having a large number of authenticated reference samples in the database this issue is diminished. However, given the international nature of the trade and the large number of factories producing glass wine bottles in the 19th century onwards, such a database would need to be very large.

One of the key methods used to date 'biological' samples has been ^{14}C dating. Naturally occurring background levels of carbon-14 amounting to 1 in 10^{12} carbon atoms are produced by cosmic ray-formation of neutrons in the earth's upper atmosphere interacting with nitrogen-14. The resulting ^{14}C reacts with oxgen to form CO_2 which is then distributed throughout global reserves comprising the atmosphere, oceans and the terrestrial biosphere. Carbon-14 has a radioactive half life of approximately 5,730 years and the amount of residual ^{14}C in sequestered CO_2 can be used to date objects in the range of 250 to 50,000 years with a precision, at best, of a few tens of years.

As a result of nuclear weapons testing during the 1950's and early 1960's the level of ^{14}C in the atmosphere increased to a level almost twice that prior to the tests. Since the Nuclear Test Ban Treaty came into effect in 1963 the ^{14}C concentration in the atmosphere has been decreasing due to, firstly, equilibration between the atmosphere, oceans and biosphere and, secondly, to dilution with CO_2 generated by the combustion of ^{14}C–free fossil fuels (Suess-effect). The shape and intensity of this 'bomb-pulse' has been determined by measuring ^{14}C in atmospheric CO_2 in both northern (*4*, *5*) and southern hemispheres (*6*, *7*) and in tree rings (*8*). This well-determined temporal change in atmospheric ^{14}C provides a clock for dating biological materials formed after 1955, and in particular, provides a far more precise method for dating the vintage of wines than that offered by measuring the radioactive decay of ^{14}C. The level of ^{14}C in the uptake and sequestering of atmospheric CO_2 by grape vines and the conversion and storage of this CO_2 into sugars and plant secondary products in the grape berry provides a memory of the year when that sequestration took place. This memory is retained when the grapes are converted into wines.

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There have been various studies on the use of carbon isotopes to authenticate the age and origin of beverages (*9*, *10*). Additionally, residual ^{14}C levels in grape-derived ethanol has been used to provide insight into temporal and spatial distribution of $^{14}CO_2$ concentrations in European countries that relate to regional and local fossil fuel emissions (*11*). However, these studies have not utilised the usefulness of both the alcohol and the non-alcoholic components in age or authenticity determinations. The measurement of the radiocarbon concentration in a single component does not necessarily provide unequivocal proof of vintage. For this reason, the unambiguous determination of a wine vintage requires the measurement of the ages of a range of wine components, the greater the number, the greater the degree of surety.

Candidate compounds for age determination include those components present in wines in higher concentration such as ethanol and grape-derived organic acids such as malic and tartaric acid. Other candidates include various phenolic components naturally present in the grape and succinic acid and glycerol produced as yeast-derived fermentation by-products of glucose.

The use of accelerator mass spectrometry (AMS) extends the applications of the bomb-pulse method by allowing the analysis of ^{14}C in samples a small as submilligram. Different AMS facilities have different sample size requirements usually in the range 0.5 – 1mg but 0.1mg carbon is not unusual. Therefore, whilst the method requires a sample to be taken from the bottle, this sample can be a small as 2ml, or less as discussed later, and can be removed at the time of topping up or re-corking of expensive wines. The small amount of sample required makes the method useful for the dating of recent biological substances, changes in atmospheric CO_2 levels and in substances of forensic significance (*12*).

Another method which uses bomb-pulse generated radionuclides measures the gamma rays emitted by ^{137}Cs. Since these gamma rays can pass through glass and given the high sensitivities of their detection this method is capable of authenticating wines pre- and post-bomb pulse without the need for opening the bottle (*13*). However, it is pointed out that the measurement of ^{137}Cs cannot be used as a chronometer like ^{14}C but determines date as a function of before and after bomb pulse due to large variations associated with geography including complications due to Chernobyl.

Materials and Methods

Twenty wines in a series of single-label wines of vintages from 1958 to 1997 were subject to fractionation and a limited subset of alcohols and tannins derived from these fractionations were measured for ^{14}C content. The wines were highlighted by being made from Barossa Valley Shiraz and made with minimal exposure to oak. The major components of alcohol, organic acids and tannins were isolated according to scheme outlined in Figure 1. Typically, 100 mL of wine was distilled under reduced pressure (maximum bath temperature 50°C, constant head temperature 34°C) to yield a low wine of approximately 40% v/v ethanol. The resulting low wine was distilled at atmospheric pressure using a

Vigreux column to yield ethanol at 95% v/v strength. Typical yields were between 6.8 and 12.0 mL ethanol.

The dealcoholised wine from the first distillation above (typically 70 mL) was treated as per Figure 2 to isolate the various phenolic compounds following the method of Oszmianski et al. (*14*) using Sep-Pak Vac 20cc (5g) C-18 reverse phase cartridges (Waters, Ireland). Organic acids were recovered alongside the phenolic acids and were subjected to futher treatment for recovery and purification (*15*). Tannins were recovered in Fraction IV by elution with methanol and after adding water the methanol was removed under vacuum and the resulting aqueous solution freeze dried to give yields of between 98 and 289 mg anhydrous tannin.

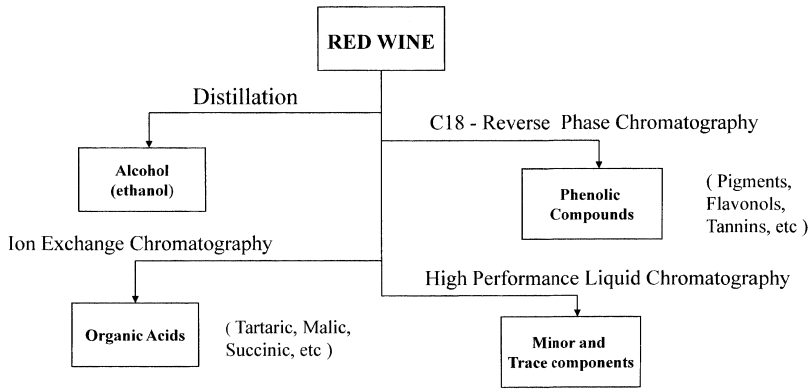

Figure 1. Schematic for the isolation of various wine components.

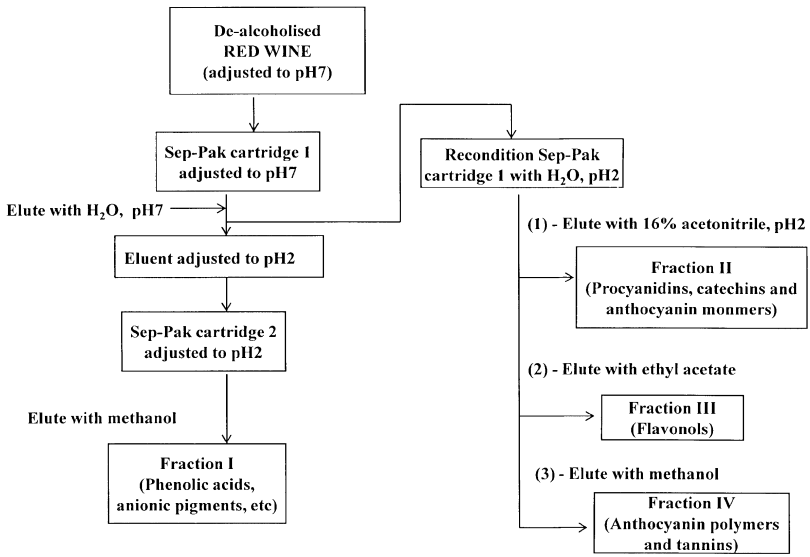

Figure 2. Protocol for the separation of wine phenolics, with specific reference to tannins, based on the method of Oszmianski et al. (14).

It is pointed out that micro distillations can be carried out on samples as small as 2 mL using commercially available micro stills and the tannin extractions and purification were also routinely carried out on 2 mL of alcohol-removed wine (*14*).

^{14}C Determinations

All samples were measured at the ANTARES AMS facility at ANSTO, Lucas Heights, New South Wales (*16*, *17*). Sample preparation relies upon the conversion of carbon in the sample into graphite by combustion to CO_2 followed by reduction of the CO_2 to graphite. Details of the method together with a full description of the AMS technique are presented by Buchholz et al. elsewhere in this volume (*18*). ^{14}C values are expressed as $\Delta^{14}C$ which measures the ratio of ^{14}C in the sample compared to the ^{14}C in a standard, taking into account any isotope fractionation that occurs during CO_2 absorption in the method of analysis or uptake by plants. $\Delta^{14}C$ values are quoted in "per mil", parts per thousand or ‰. In 1955, prior to the impacts of atmospheric atomic bomb testing the $\Delta^{14}C$ value in the southern hemisphere was -10‰. This rose to 695‰ in 1965, after the atmospheric tests, and has fallen to 75‰ by 2005 (*7*).

Results and Discussion

$\Delta^{14}C$ concentrations for the alcohols derived from the vintage wines and several tannin samples are listed in Table 1 and plotted in Figure 3. Calibrated age ranges were determined using the Southern Hemisphere bomb pulse curve (*19*) and the 'calibrate' calibration software (*20*), which allows the use of negative radiocarbon ages. In Figure 3 the measured $\Delta^{14}C$ concentrations are plotted at Jan 1st, the midpoint of the growing season in the Southern Hemisphere. The agreement between the predicted vintage year based on the measured ^{14}C results with the vintage indicated on the bottle label is very good although there are some minor discrepancies possibly due to the legal top-ups discussed before and to regional differences in $^{14}CO_2$ atmospheric levels. Of particular interest is year 1969, which was a poor vintage with a particularly wet growing season resulting in a lesser wine quality.

The 1969 vintage wine in the series measured shows a ^{14}C concentration typical for the 1964 or 1975 growing season. For that particular wine, the calibrated age for the tannin fraction corresponds to September 1963 ± 2 months or January 1974 ± 4 months. Usually, red wines have tannins added to them during the winemaking process. These tannins can come from the heartwood of trees, from galls and from grape seed. The use of heartwood, which is typically many years old, would result in a tannin ^{14}C concentration corresponding to the pre bomb pulse period. Galls and grape seed tannins on the other hand predominantly originate from the previous vintage and thus should result in a "1 year older" ^{14}C concentration. Furthermore, red wines can also pick up oak tannins from the barrels they are stored in. For this study we selected wines that had little contact with oak in that they were stored in very old barrels and were prepared with tannins principally derived from galls and grapes. Knowing that

the wine under investigation was prepared using tanning derived from galls and grapes we can conclude that it is compatible with a 1975 vintage, and not 1969 as shown on the label. Measurements for two other Barossa Valley Shiraz wines of 1969 vintage gave alcohol measures corresponding to February and July 1969 in agreement with their labels. Tannin measures corresponding to September 1967 and January 1968 support the label authenticities of these wines.

It is noted that the atmospheric $^{14}C/C$ ratio is decreasing exponentially with an exponential constant of approximately 18 years (*7*). Presently, the annual differential in $\Delta^{14}C$ is at around 5 ‰ / year, a factor of 2 above the precision level for the ^{14}C AMS technique. Due to seasonal variations being larger than the magnitude of this decreasing trend, dating to the nearest year of wine vintages made after 1990 is very difficult. Never-the-less, any value greater than the 1955 level indicates that the vintage of the wine is post bomb-pulse. Furthermore, as the changes of "in-bottle" ^{14}C concentrations as a result of radioactive decay of ^{14}C is negligible, the ^{14}C vintage fingerprint of wines from 1955 to about 1990 will be valid for many hundreds of years into the future.

As mentioned earlier, in order to carry out the AMS ^{14}C dating a small amount of wine needs to be withdrawn from the bottle. The size of this sample is ultimately determined not by the sensitivity of AMS but by sample fractionation and preparation ready for AMS. Whilst companies producing icon wines in Australia offer a service to top up and recork their wines given various quality and age criteria, providing an opportunity for wine to be available for analysis, not all wine companies may provide this service. For very old wines in particular, there is the concern that the wine may not be original in the first case and in countries such as France the law dictates that the top-up wine has to be of the same blend (same wine) and age as the wine being topped up.

An alternate method using bomb-pulse generated ^{137}Cs overcomes the need for sampling the wine. Hubert and collegues (*13*) have developed a technique for authenticating wines based on detection of the gamma rays from radioactive decay of ^{137}Cs. The key to the method is the development of a low background gamma–ray spectrometers and the fact that these gamma rays penetrate glass and cork. Using such instruments levels of radioactivity from ^{137}Cs in wines can be dectected down to a few mBq/L in processed samples and a few tens of mBq/L in intact, unopened bottles of wine. This is highly significant given that levels of ^{137}Cs in wines have been detected in excess of 1000 mBq/L (see Figure 4). ^{137}Cs produced by atmospheric nuclear explosions provides both a pool in the upper atmosphere and over time some of this material has been deposited on earth in the form of dust and rain.

Table 1. Measured $\Delta^{14}C$ values for alcohol and tannin components isolated from vintage wines. Alcohol and tannin measures were obtained from additional wines from the 1969 vintage for comparison (n*).

Sample Type	*Year (bottle label)*	*$\Delta^{14}C$ [‰]*	*Calibrated age range*
Alcohol	1958	69.9 ± 4.6	Feb 1958 ± 3 months
Alcohol	1963	248.6 ± 4.7	Sep 1962 ± 3 months
Alcohol	1965	609.7 ± 5.6	Jan 1965 ± 4 months
Alcohol	1966	575.9 ± 6.5	Jun 1964 ± 2 months Dec 1967 ± 3 months
Alcohol	1968	553.7 ± 6.5	Sep 1968 ± 5 months
Alcohol	1969	389.4 ± 6.3	Aug 1963 ± 2 months Feb 1975 ± 6 months
Tannin	1969	419.5 ± 4.9	Sep 1963 ± 2 months Jan 1974 ± 4 months
Tannin	1969[1]*	585.5 ± 5.9	Sep 1967 ± 3 months
Alcohol	1969[1]*	540.4 ± 6.0	Feb 1969 ± 7 months
Tannin	1969[2]*	574.0 ± 5.8	Jan 1968 ± 3 months
Alcohol	1969[2]*	530.8 ± 6.0	Jul 1969 ± 7 months
Alcohol	1971	491.8 ± 7.0	May 1971 ± 7 months
Alcohol	1973	463.2 ± 4.7	Sep 1972 ± 4 months
Alcohol	1974	448.3 ± 5.5	Feb 1973 ± 4 months
Alcohol	1978	336.8 ± 7.5	Mar 1977 ± 9 months
Alcohol	1980	300.8 ± 4.6	Apr 1979 ± 5 months
Alcohol	1982	240.3 ± 6.7	Oct 1982 ± 8 months
Alcohol	1985	214.9 ± 4.6	Sep 1984 ± 9 months
Alcohol	1989	174.0 ± 4.6	Aug 1988 ± 13 months
Alcohol	1991	160.8 ± 5.2	Apr 1990 ± 17 months
Alcohol	1993	141.3 ± 3.6	Nov 1991 ± 15 months
Alcohol	1995	117.0 ± 3.8	Mar 1995 ± 11 months
Alcohol	1997	120.6 ± 4.3	(outside calibration)

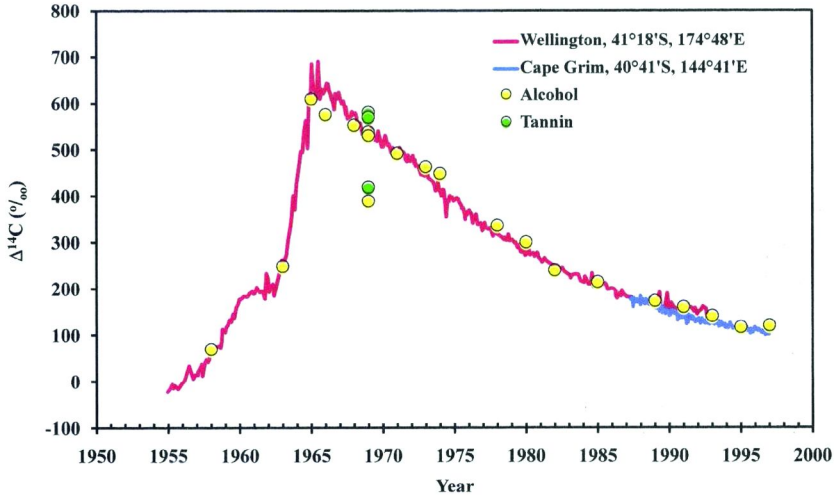

Figure 3. Comparison between Δ14C values for alcohol and tannin components extracted from wines and the compiled Southern Hemisphere atmospheric 14C (6, 7). (see color insert)

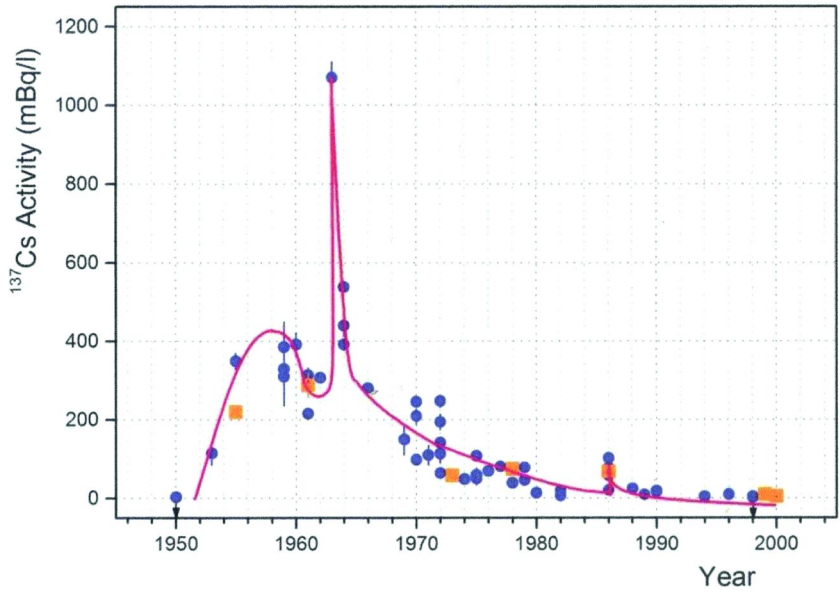

Figure 4. Activity of 137Cs (mBq/L) found in Bordeaux wines normalized to 1st January 2000. Solid blue circles correspond to measurements of ashed samples whilst the orange squares correspond to in-bottle (non-destructive) samples. The solid line represents a reference curve for the data (taken from reference (13) with permission). (see color insert)

Local concentrations will vary depending on wind patterns and local rainfall and the amount present in wines will be determined by grapes contaminated by dust containing ^{137}Cs entering the winery and by uptake of ^{137}Cs into the grape vine by absorption through the leaves (*21*). The amount of ^{137}Cs measured at a particular date will also be determined by radioactive decay of ^{137}Cs which has a half-life of approximately 30 years.

This provides a method for authenticating the age of wines in that for a particular vintage a wine must have a matching ^{137}Cs activity. This is true irrespective of the amount of radioactive decay of ^{137}Cs as a function of time. Figure 5 shows the projected levels of ^{137}Cs in Bordeaux wines at vintage and projected to the year 2050. The projected data indicate that it will be year 2100 before the ^{137}Cs levels in wine drops below 10mBq/L, well above the level of detection, from the 138 mBq/L introduced as a result of the 1986 Chernoble nuclear incident.

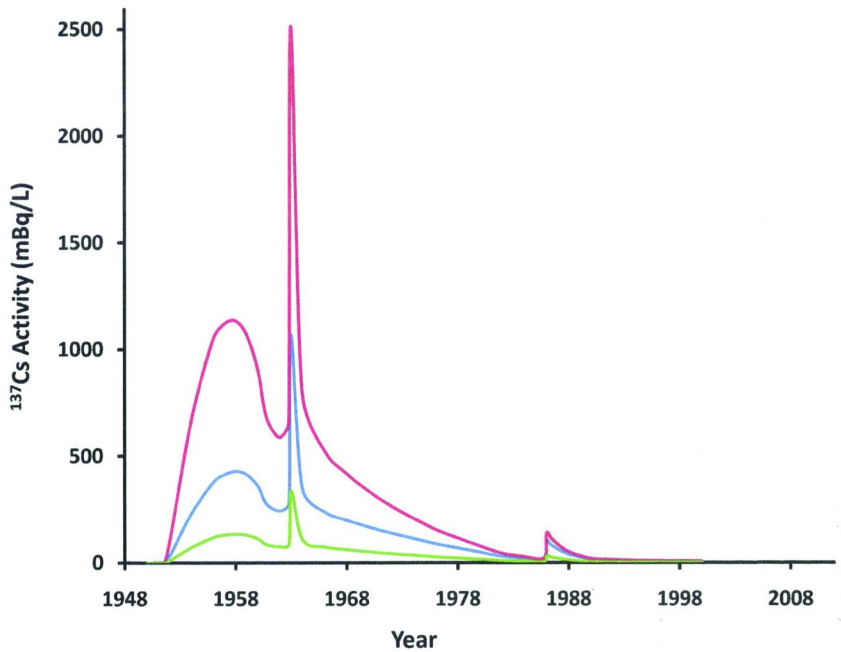

Figure 5. Projected activities of ^{137}Cs (mBq/L) in Bordeaux wines as a function of radioactive decay based on a half life of 30 years. (—) data normalized to 1st January 2000; (—) at the time of vintage; (—) expectation values in the year 2050 (based on data presented in reference (13)). (see color insert)

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Conclusions

All of the techniques used for wine vintage authentication mentioned rely on having an appropriate database to compare test samples against those of authenticated references. In particular, those methods that are based on large and frequent changes in chemical composition of wine or container will require commensurately larger databases to be useful. In the case of bomb-pulse ^{14}C authentication, the method is made more robust by the simultaneous analysis of a range of wine components, the age of each component needing to agree with each other. This reduces the size of the database required and should theoretically need only the available atmospheric ^{14}C measurements for its implementation. On the other hand, the method requires a physical sample to be removed from the bottle which may create problems such a bottle integrity. Other methods such as the measurement of 137Cs in the wine may not provide the precision in age of the wine but can be used without compromising the integrity of the wine within the bottle.

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Chapter 7

Overview of Chemical Markers for Varietal Authentication of Red Wines

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Wine has an economic value that is associated with a luxury product, and its consumers demand reliable information. Additionally, a global wine market needs objective tools to verify varietal authenticity, making international trade more transparent. In this context, useful analytical methods for wine identification are needed to protect quality wines by preventing their illegal adulteration. The present work provides an overview of the published literature about chemical markers that has been used to classify red wines according to their variety. It also discusses the data analysis methods, as well as the incidence of wine processing technology and other practical aspects that can limit the applicability of proposed alternatives.

Introduction

Wine has an economic value that is associated with a luxury product, which is subject to different trends. Wine consumer preferences can change over time due to different factors, i.e. type of consumers, traditions, trends and marketing, image of origin area, the opinion of wine writers. For example, the movie "Sideways" acted as driving force, increasing demand for Pinot Noir and diminishing demand for Merlot wines (*1*, *2*). Consumers demand reliable information, and when they choose a wine, the winery's reputation is more influential in the decision than the product characteristics (*3*).

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Additionally, a global wine market needs objective tools to verify varietal authenticity, making international trade more transparent. In this context, analytical methods can be used for wine identification, thus protecting quality wines and preventing their illegal adulteration (*4*).

The present work provides an overview of the published literature about the chemical markers (i.e. anthocyanins, flavonoids, shikimic acid) that have been proposed to classify red wines according to their variety. The paper also discusses the data analysis methods as well as the incidence of wine processing technology and other practical aspects that can limit the applicability of proposed alternatives.

Chemical Markers

Phenolic Compounds

Phenolic acids are a subclass of a larger category of metabolites commonly referred to as "phenolics", which can be classified into polyphenols and simple phenols (*5*). Red wines include a number of phenolics constituents, such as anthocyanins, flavan-3-ols, flavonols and phenolic acids (*6*). Phenolics are a large and complex group of metabolites that particularly contribute to the sensorial properties of grapes and red wines and are responsible for red wine color, flavor, astringency and bitterness (*7*, *8*).

Anthocyanins are the most characteristic colored compounds in red wines, and it is known that the anthocyanins profiles are determined by genetic factors and that their distribution varies considerably among different grape cultivars (*9*, *10*). Anthocyanin profiles for each variety are relatively stable, while absolute concentrations can vary widely between different vintages, due to both environmental factors such as light, irrigation, and temperature (*11–14*).

The anthocyanin profile has been used to classify red wine according to their grape variety (*15–20*). Some propose to use simple ratios between acetylated and coumaroylated anthocyanins (Rac/cum) alone (*16*, *20*) since the acyl and coumaroyl forms are more stable during the winemaking process and ageing (*21*). Cabernet Sauvignon wine recorded the highest values of acyl and coumaroyl anthocyanins, Merlot presents higher content in peonidin and coumaroyl anthocyanins (*22*), and Pinot Noir does not have acylated anthocyanins (*23*, *24*). However, red wine classification using only the anthocyanin profiles is not sufficient to discriminate between a large numbers of red wine varieties.

Flavonols are a class of flavonoid found in grape skins. They are yellow pigments that contribute directly to the color of white wines, but in red wines they are masked by anthocyanin (*25*). In addition, flavonoids have been identified as one of the best phenolics with antioxidant activity in wine (*26*). Red wine also contains organic acids, which are product of sugar oxidation or of alcoholic fermentation during the winemaking process (*27*).

Shikimic acid is another carboxylic acid that comes from grape skin and is always present in musts and wines (*28*). It is an intermediate molecule produced in the shikimate pathway, the biosynthetic pathway of the flavonoids present in the grape (*18*, *29*). Shikimic acid (*18*, *30*) and flavonols (*31*, *32*) can be used for varietal differentiation of red wines.

Table I presents the differentiation of red wine varieties achieved with determination of wine polyphenols in HPLC, CE or UV-vis spectrometric measurements.

Burns et al. (*33*) has questioned the use of the anthocyanin ratio because it insufficiently discriminates Cabernet Sauvignon wines from hybrid grape varieties. The process is inconclusive for wines that contain a percentage lower than 75% in America (*34*) and 85% in Europe (*35*) of the variety, which is the limit allowed by legislation for labeling a wine as monovarietal.

Table I. Red wine varieties classified using polyphenolic compounds

Separated wine varieties (Number of samples)	*Chemical marker*	*Ref.*
B. Portugieser (7) / B. Spätburgunder (31) / Domina (7) / Frühburgunder (1) / Schwarzriesling (3) / Zweigeltrebe (2) / Dornfelder (1)	Anthocyanin composition	(*17*)
C. Sauvignon (30) / Merlot (6) + Carmenère (6)	Shikimic acid	(*30*)
C. Sauvignon (231) / Merlot (76) /Carmenère (96)	Rac/cum, shikimic acid, flavonols	(*31*)
C. Sauvignon (127) / Merlot (60) / Carmenère (68)	Rac/cum, shikimic acid, log Pe%	(*32*)
B. Portugieser (7) / B. Wildbacher (7) / St. Laurent (7) / B. Zweigelt (15) / Blaufränkisch (7) / Blauburger (2)	Organic acids, flavonols, flavanols, resveratrol	(*39*)
Tannat (6) / C. Sauvignon (8) / Merlot (8)	Anthocyanin composition	(*40*)
C. Sauvignon (80) / Merlot (35) / Carménère (57)	Phenolic compounds	(*41*)
Graciano (3) / Tempranillo (4) / Garnacha (4) / Cariñena (4) / Merlot (4) / C. Sauvignon (4)	Anthocyanin composition	(*42*)
C. Sauvignon (13) / Merlot (10) / Syrah (11) / Pinotage (11) / Ruby Cabernet (10)	22 non-coloured phenolic compounds	(*43*)
Monastrell / Tempranillo / C. Sauvignon / Graciano / Cinsault Noir	Organic acids and color parameters	(*44*)
Tempranillo (11) / Garnacha (6) / Monastrell (11) / Bobal (10) / C. Sauvignon (6)	Anthocyanin composition	(*45*)
Agiorgitiko (6) / C. Sauvignon (7) / Merlot (5) / Syrah (9) / Xinomavro (8)	Hydroxycinnamate derivatives, flavanols, flavonols and anthocyanins,	(*46*)

Cliff et al. (*24*) evaluated the varietal differentiation of 78 commercial red wines: Cabernet Sauvignon, Cabernet Franc, Merlot, and Pinot Noir, from Okanagan Valley, BC, Canada, vintages from 1995 to 2001. Pinot Noir wines were located into distinct groups and there is much dispersion in the other varietal groups. Possible coupage mixes made within the proportion, so the wine can be labeled as mono-varietal wines, could explain this result.

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Another approach is the spectroscopic analysis of wines in the spectral regions in order to gather the information of all polyphenolic compounds in a profile. Then multivariate analysis can be used for the varietal classification of wines. An author has used MIR spectroscopy to discriminate between different varietals of red wines (Table II). ^{1}H NMR spectroscopy can be used to differentiate between Agiorgitiko and Mandilaria red wine (*36*). Cozzolino et al. (*37*) scanned wine bottles in the Vis-NIR region (wave numbers 600–1,100 nm) in a monochromator instrument in transflectance mode. Principal component analysis (PCA) and partial least squares (PLS) regression were then used to interpret the spectra and develop calibrations for wine composition. Cuadros-Inostroza et al. (*38*) used ultra high performance liquid chromatography coupled to ultra high-resolution mass spectrometry (UPLC-FT-ICR-MS), to obtain a metabolomic profiling of wine samples. The authors obtain the mass spectra in positive and negative ionization modes; then with spectral analysis, they gather a characteristic fingerprinting of the analyzed wine. These methods was used to classify Cabernet Sauvignon, Merlot, Carménère and Syrah wines according to their variety, origin, vintage, and quality. These methods are fast and do not require sample pretreatment.

Table II. Red wine varieties classification using spectroscopic measurements

Separated wine varieties (Number of samples)	*Chemical marker Spectral region*	*Ref.*
C. Sauvignon (50) / Merlot (16) / Shiraz (53)	926 to 5012 cm−1.	(*47*)
Agiorgitiko (20) / Xinomavro (8) / Merlot (6)	1800–900 cm−1	(*48*)
C.Sauvignon (8) / Blaufränkisch (6) / Merlot (3) / Pinot Noir (8) / St. Laurent (7) / Zweigelt (6)	1640 to 950 cm−1, 250 and 600 nm	(*49*)

Aromatic Compounds

Wine aroma can be influenced by grape variety, climate, soil, fermentation condition, yeast strains and production process (*50–54*). The aromatic profile and the compounds, such as terpenes and norisoprenoids, are strictly related to the varietal characteristics in wines (*55*).

Mateo and Jiménez (*56*) indicated that aromatic compounds, such as monoterpenes, can only differentiate between intensely flavored Muscat and non-Muscat; the total monoterpene concentration can be used to differentiate between aromatic and neutral wine varieties, but they cannot be used to classify them into different grape varieties. Also the authors pointed out that the number of samples used in these studies is insufficient and do not provide the diversity needed to account for all the possible influences in the monoterpene concentration (*57*). Such is also the case of Piñeiro et al. (*58*), in which a group of 23 wines of 13 varieties of white and red wines were analyzed and classified by SPE-GC, SPME-GC and principal component analysis. The result could only aggregate the samples into three groups: aromatic, red and white wines; the terpenic

compounds terpineol, linalool, geraniol and citronellol were the most influential in correct separation. Wine classification using aromatic compounds is more successful for white wine varietal discrimination (*55*, *59*). Table III presents some red wine varieties classified using volatile chemical markers measured by gas chromatography.

In grapes, volatile compounds, like alcohols, esters, acids, terpenes, ketones and aldehydes, have been useful to discriminate between red grape varieties (*31*, *57*, *60*, *61*).

Table III. Red wine varieties classified using volatile chemical markers measured with gas chromatography

Separated wine varieties (Number of samples)	*Chemical marker*	*Ref.*
C. Sauvignon (13) / Merlot (13) / C. Gernischt (13)	Isobutyl acetate, 1-Pentanol, (+,-)2,3-Butanediol, 1-Hexanol, Ethyl hexanoate, Diethyl butanedioate, Ethyl octanoate, Ethyl nonanoate, Ethyl 2-hydroxybenzoate, Ethyl decanoate, Ethyl dodecanoate	(*60*)
C. Sauvignon (7) / Merlot (7) / Pinotage (6) / Ruby Cabernet (5) / Shiraz (13) / blend (5)	Alcohols, acids, esters, phenols, aldehydes, ketones, and lactones	(*62*)
C. Sauvignon (82) / Tempranillo (66) / Merlot (61)	Organic acids (isobutyric, butyric, hexanoic and octanoic), 3-methylthio-1-propanol	(*63*)
C. Sauvignon / Tempranillo / Monastrell / Bobal (91)	Total acidity, cis-3-hexenol, methanol, glycerol, 2,3-butanediol, isobutyric alcohol, 1-pentanol, acetaldehyde, ethyl propionate, ethyl decanoate, and γ-butyrolactone	(*64*)

Data Analysis Methods

Data analysis methods for authentication purposes have been developed mainly outside the field of statistics, and most are exploratory techniques designed to deal with a multivariate data set. The simplest method is the use of ratios between proportions of different marker compounds (*18*, *20*), although this approach is empirical and completely arbitrary. With advances in analytical instrumentation, a more complex data set is generated, thus requiring new tools to process and interpret all the information for wine sample classification.

Authentication problems are classification problems, and thus classification methods are needed. There are two categories of classification methods, supervised and unsupervised. In supervised classification methods, the samples in the training set are labeled according to a class e.g. variety, origin and vintage. In unsupervised classification methods, the samples in the training set do not have a specified class. The most common supervised classification method used

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for varietal classification in wines is linear discriminant analysis (LDA), its variations such as quadratic discriminant analysis (QDA), or combined stepwise for variable selection purposes. LDA has been used by various authors (*44*, *46*, *47*, *52*, *54*, *57*, *62*); some authors combined LDA with stepwise (*9*, *42*, *60*) and discriminant canonical analysis (*24*, *39*, *40*). Other methods for supervised classification used in wines include artificial neural networks (*4*, *41*) and partial least-squares discriminate analysis (PLS-DA) (*36*, *37*). Unsupervised methods, such as principal component analysis (PCA) (*9*, *10*, *17*, *36*, *37*, *42–44*, *47*, *50*, *59*, *60*, *62*, *63*, *65*) and cluster analysis (*43*, *62*), are the most commonly used for pattern recognition in wine classification.

Probabilistic modeling, for discrimination and authentication purposes, was proposed by Brown et al. (*66*), who used Bayesian statistical methods to discriminate 39 microbiological taxa using their reflectance spectra. Probabilistic modeling has not been used for wine authentication. The work of (*67*) was applied in authentication of meat and olive oils and the work of (*68*) was applied in authentication of honey.

In a probabilistic modeling approach for food authentication, we need to assume a probability distribution function for the response vector. The response vectors are the measurements for wine samples, e.g. log-proportions of anthocyanin or concentrations of any compound; as with covariates, we can use variety, origin, vintage, technology of processing, etc. For example, if we use a normal multivariate distribution for the response vector, the mean and covariance-variance matrix for the normal distribution only need to be estimated. Then, using the Bayes rule (*69*), the probability that a wine sample comes from a specific grape variety can be estimated. Finally, with the above probabilities, each wine sample can be assigned to a grape variety using the zero – one loss function (*70*), which classifies the sample in the grape variety for which the probability is bigger.

The advantage of the probabilistic approach is that covariates can be introduced in the model, which could improve the classification results. Its disadvantage is that it is not known enough outside the statistic field.

Incidence of Wine Processing Technology

Polyphenols used in varietal differentiation are extracted from wine grapes during the first winemaking stages. It has to be considered that any process that can modify the extraction of flavonoids or organic acids can alter the ratios and proportions used for varietal differentiation. The vinification techniques of thermovinification with skins, maceration time, and enzyme treatments and the different ageing processes must also be considered. Budíc-Leto et al. showed the effects of winemaking techniques on the anthocyanin content of specific Croatian wines made from Babić (*Vitis vinifera*, L.). Winemaking processes and reactions that take place during maturation significantly influence anthocyanin and proanthocyanidin content in wine. Prolonged maceration duration caused a decrease in the anthocyanin content in young wine (*71*). Later, they presented results, indicating that the maceration temperature was more influential in the

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final anthocyanin concentration than the duration of the maceration, and that the different treatments were more influential in some anthocyanins than others (*72*).

Fisher et al. 2007 showed the effect of different vinification technologies, such us enzyme treatment, thermovinification and duration of skin contact, on the acylated/non acylated and acetylated/coumaroylated anthocyanins of red wines made from Dornfelder and Portugieser cultivars. Increasing the temperature during thermovinification enhanced the ratio of acetylated/coumaroylated anthocyanins. Prolonging skin contact during fermentation on the skins induced higher ratios of acetylated/coumaroylated anthocyanins. Assessment of several commercial pectinases revealed significant acetyl- and/or coumaroyl esterase side activities. Some of the evaluated wines were outside of the authenticity confidence intervals of the acylated/nonacylated and acetylated/coumaroylated ratios (*73*).

Gonzales-San José et al. showed the varietal differentiation of Tempranillo, Garnacha, Monastrell, Bobal and Cabernet Sauvignon red wines by anthocyanin profiling and multivariate analysis. The classification was not affected by the different winemaking methods, such as claret, carbonic maceration and "double paste" (double pomance), in comparison with the classification obtained with the wine made with the traditional method (*45*).

In contrast, phenolic and volatile compounds seem to be more influenced by the type of winemaking technologies. Spranger et al. (*65*) showed that winemaking technologies, such as carbonic maceration, stem-contact fermentation and non stem-contact fermentation, in Castelão red wines produced changes in phenolic and volatile composition. These changes in the composition of phenolics and volatile compounds were useful to distinguish the skin fermentation wines from the wines made by the different winemaking technologies. The reliability of these compounds for varietal differentiation under different vinification techniques could be questionable.

Cadahia et al. (*74*) observed the effect of oak barrel ageing over Tempranillo, Cabernet Sauvignon and Merlot monovarietal wines. Low molecular weight phenolic and volatile compounds were evaluated over a period of 3, 6, 9 and 12 months of ageing. At the end of the study, the three wine varieties could be differentiated, although the groupings changed over time, moving in the plane of the canonical discriminant analysis. This trend is consistent with our own results for Cabernet Sauvignon, Merlot and Carménère wines produced in Chile.

Conclusions

The present overview confirms that chemical marker profiles, characteristic of the respective grape variety, are transferred to wine during the winemaking process, and they can serve as a basis for a red wine classification system according to their grape variety.

For varietal differentiation of red wines, the preferred chemical markers are flavonoids, shikimic and phenolic acids. These compounds have been used, alone or in combination, to generate profiles to discriminate between red wine varieties.

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To identify patterns or classification groups for varietal differentiation, more complex data analysis methods, like PCA or LDA, are more reliable than certain simple ratios of specific anthocyanins.

Although processing technology can slightly affect these marker profiles, the system is more robust if different marker groups are used together and combined with appropriate data analysis methods.

Other developments, such as direct spectroscopic wine analysis in the IR range or very recently described UPLC-FT-ICR-MS, open interesting perspectives to differentiate red wine varieties but further research and development of broader data bases are required in both cases.

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Chapter 8

Flavonol Profiles for Grape and Wine Authentication

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Flavonols are a class of flavonoid compounds with a potential use as chemical markers for cultivar authentication of grapes and their wines, like anthocyanins do in the case of red grape and wine. Flavonols have the advantage of occurring in both red and white grapes and wines. Red grape flavonols are 3-*O*-glucosides, 3-*O*-galactosides, and 3-*O*-glucuronides of six flavonoid structures (kaempferol, quercetin, isorhamnetin, myricetin, laricitrin, and syringetin). In contrast, white grapes do not contain any flavonol derived from myricetin, laricitrin and syringetin. In addition, grapes usually contain traces of rutin (quercetin 3-*O*-rutinoside). Grape flavonol profiles are characteristic enough to allow statistical differentiation among grape cultivars. In wine, the original grape flavonol 3-*O*-glycosides suffer partial hydrolysis and the use of flavonol profiles for cultivar authentication only lead to good results after expressing the results as aglycone-type flavonol profiles.

Introduction

Flavonols are a class of flavonoid phenolics located in *Vitis vinifera* grape berry skins, where they are involved in UV screening (*1*, *2*), with their biosynthesis being light-dependent (*3*). The so-called "teinturier" grape cultivars are the only ones known to contain flavonols, together with anthocyanins, in both the skin and the flesh of berries (*4*). Regarding the color of the wines, flavonols are yellow

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pigments which contribute directly to the color of white wines, but in red wines flavonols are masked by anthocyanins, the red pigments. However, flavonols affect red wine color by means of copigmentation (*5*, *6*). In addition, flavonols have been identified as one of the best phenolics with antioxidant activity in wine, especially in white wines (*7–9*).

Phenolic compounds biosynthesis in *Vitis vinifera* grapes is under genetic control and the differences among grape cultivars are sometimes great enough to use the phenolic composition of the grape as a tool for cultivar authentication and differentiation. In the case of red wine grape cultivars the anthocyanin profiles have been widely used for cultivar authentication purposes. Flavonol profiles have also demonstrated some ability for cultivar differentiation for both red and white wine grape cultivars although the results have not always led to consistent conclusions (*10–17*). Perhaps one of the reasons for the inconsistency shown by the data concerning grape flavonol profiles was the scarce knowledge of such grape phenolics in contrast to the very well-known grape anthocyanins. Over the last three years, our group has contributed to the identification of the complete pool of flavonols present in grapes (*12*, *18*, *19*) which are depicted in Figure 1.

Flavonols of *Vitis vinifera* red grape cultivars occur as three glycosylated series (3-*O*-glucosides, 3-*O*-galactosides, and 3-*O*-glucuronides) of the six possible flavonoid structures, according to the B-ring substitution pattern (kaempferol, quercetin, isorhamnetin, myricetin, laricitrin, and syringetin). Rutin (quercetin 3-*O*-(6″-rhamnosyl)-glucoside or quercetin 3-*O*-rutinoside) had been erroneously identified as an important grape flavonol (*20*), but is currently considered a minor compound (*18*, *19*, *21*). Non-vinifera red grape cultivars seem to contain the same aforementioned flavonol 3-*O*-glycosides (*22*).

With regards to white grape cultivars, the presence of isorhamnetin-type and myricetin-type flavonols had been considered exclusive to red grape cultivars (*23*). However, the occurrence of isorhamnetin-type flavonols in the skin of white grapes had already been suggested (*15*, *16*) and, recently, ESI-MS^n data supported the occurrence of the 3-*O*-glucosides and 3-*O*-galactosides of kaempferol, quercetin, and isorhamnetin, and also the 3-*O*-glucuronides of kaempferol and quercetin in a wide collection of white grape cultivars (*19*); in addition, traces of isorhamnetin 3-*O*-glucuronide and rutin were found in some cases.

Flavonol profiles of wines differ from those of their original grapes because of the hydrolysis of flavonol 3-*O*-glycosides (*12*). Therefore, wine flavonol profiles consist of a mixture of flavonol 3-*O*-glycosides and free flavonol aglycones released from them. The causes behind flavonol 3-*O*-glycosides hydrolysis in wine have not yet been revealed and preliminary results obtained by our group do not clearly suggest the possible implication of the acidity of wine or the glycosidase activities of some enological enzymes used during winemaking. However, the knowledge accumulated by our group permit us to suggest that flavonol 3-*O*-glycosides hydrolysis develops indistinctly in both young and aged wines; in addition, the degree of hydrolysis seems to be dependent on the flavonoid structure and also the kind of 3-*O*-glycoside (*12*).

In this work, we report on the use of flavonol profiles for cultivar authentication and differentiation of both grapes and wines. On the one hand, we show practical applications on different red and white grape cultivars. In the case

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of white grape cultivars, flavonol 3-*O*-glycosides are practically the only available phenolics which can be used for the aforementioned purpose. On the other hand, we have tested the use of flavonol profiles for cultivar authentication and differentiation of red wines, paying special attention to the limitations imposed by the partial hydrolysis of flavonol 3-*O*-glycosides.

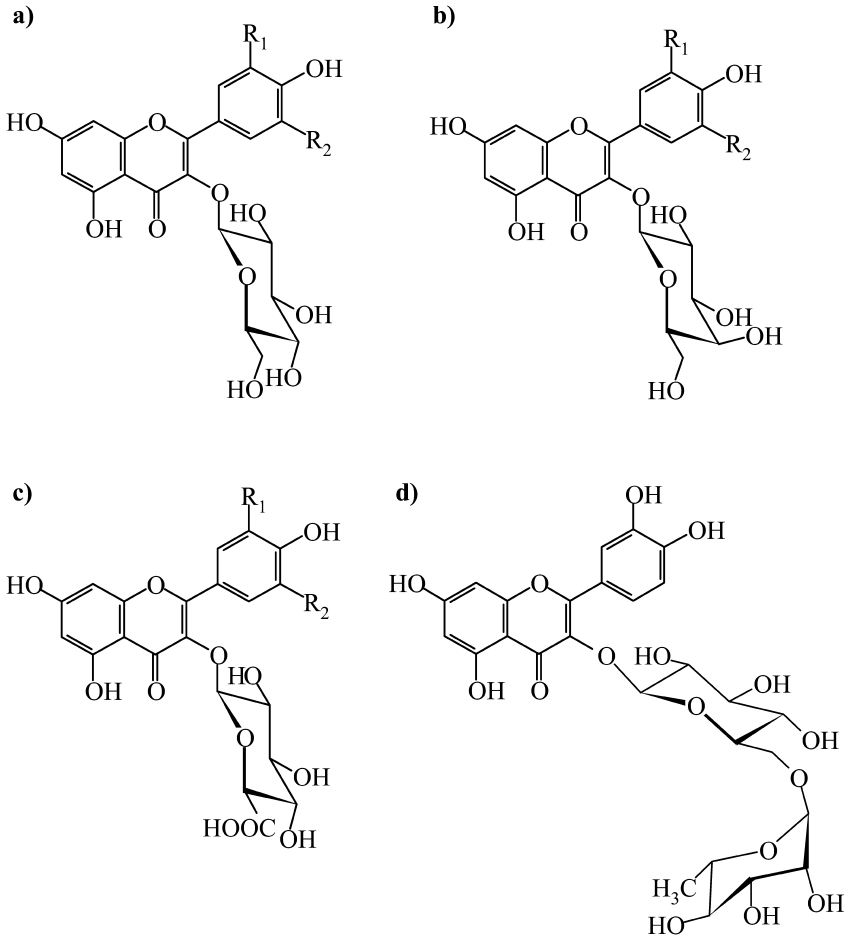

Figure 1. Flavonol 3-O-glycosides identified in Vitis vinifera grapes: a) flavonol 3-O-glucosides; b) flavonol 3-O-galactosides; c) flavonol 3-O-glucuronides; d) rutin (quercetin 3-O-(6''-rhamnosyl)-glucoside). Flavonoid structures according to B-ring substitution pattern: kaempferol ($R_1 = R_2 = H$); quercetin ($R_1 = OH$, $R_2 = H$); isorhamnetin ($R_1 = OCH_3$, $R_2 = H$); myricetin ($R_1 = R_2 = OH$); laricitrin ($R_1 = OCH_3$, $R_2 = OH$); syringetin ($R_1 = R_2 = OCH_3$).

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Experimental

Chemicals and Grape and Wine Samples

All solvents were of HPLC quality and all chemicals of analytical grade (> 99%). Water was of Milli-Q quality. Available commercial standards of flavonol 3-*O*-glycosides were from Extrasynthese (Genay, France): 3-*O*-glucosides of quercetin, kaempferol, isorhamnetin and syringetin, 3-*O*-galactosides of quercetin and syringetin, and rutin. Other non commercial flavonol standards (myricetin 3-*O*-glucoside, myricetin 3-*O*-galactoside, quercetin 3-*O*-glucuronide, laricitrin 3-*O*-glucoside, and kaempferol 3-*O*-glucuronide) were kindly supplied by Dr. Ulrich Engelhardt (Institute of Food Chemistry, Technische Universität Braunschweig, Germany) or were isolated in our previous work (*18*).

Healthy *Vitis vinifera* grapes grown in the experimental vineyard (drip-irrigated vines using bilateral Royat cordon trellis) of the Instituto de la Vid y el Vino de Castilla-La Mancha (IVICAM) were collected at optimum ripeness for harvesting. The sampling was randomly carried out by picking berries from the top, central and bottom parts of the cluster, following a zigzag path between two marked rows of ten vines. We tried to sample berries from both exposed and shaded clusters by picking berries of 4-5 clusters per vine. The size of the sample was around 200 berries, which were bulked and separated into 2 sub-samples of approximately 100 berries. Before HPLC analysis, flavonols from grape skins were isolated, as described in the next section.

All the analyzed wines were commercial samples. The wine flavonols can be analyzed without previous isolation being necessary. Only red wines subjected to Origin Denomination control with a declaration as "single-cultivar wine" were selected; a control which implies that at least 85% of the wine has to be made from grapes of the same cultivar.

Isolation of Grape Skin Flavonols Fraction

An amount of 100 g of healthy grapes was finger pressed to remove the pulp and the seeds. The remaining skins were washed three times in water (Milli-Q) and softly dried twice by drying carefully them between sheets of filter paper. The dried skins were extracted with 100 mL of a mixture 50:48.5:1.5 (v/v/v) of CH_3OH/H_2O/HCOOH, using a homogenizer (Heidolph DIAX 900) for 2 min and then centrifuging at 2500g at 5 ºC for 15 min. A second extraction of the skin pellets yielded nearly 99% of the grape skin phenolic content, as confirmed by HPLC of successive extractions (up to five). The combined supernatants were stored at -18 ºC until use. White grape skin extracts can be analyzed without isolation of their flavonol fraction.

Anthocyanin-free flavonol fractions from red grape skin extracts were isolated following the procedure previously described (*12*) using SPE cartridges (Oasis MCX cartridges, Waters Corp., Mildford, MA; cartridges of 6 mL capacity filled with 500 mg of sorbent). The eluate containing flavonols was dried in a rotary evaporator (40 ºC) and re-solved in 25% methanol for injection.

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Analysis of Flavonols by HPLC-DAD-ESI-MS^n

HPLC separation, identification and quantification of flavonols was performed on an Agilent 1100 Series system (Agilent, Germany), equipped with DAD (G1315B) and LC/MSD Trap VL (G2445C VL) electrospray ionization mass spectrometry (ESI-MS^n) system, and coupled to an Agilent Chem Station (version B.01.03) data-processing station. The mass spectra data was processed with the Agilent LC/MS Trap software (version 5.3). The samples of grape flavonol fractions and wines were filtered (0.20 µm, polyester membrane, Chromafil PET 20/25, Macherey-Nagel, Düren, Germany) previous to the injection (50 µL) on a reversed-phase column Zorbax Eclipse XDB-C18 (4.6 x 250 mm; 5 µm particle; Agilent, Germany), thermostatized at 40 ºC. We used a previously described chromatographic method specifically developed for the analysis of grape and wine flavonols (*18*). For identification, ESI-MS^n was used in both positive and negative modes, setting the parameters as formerly described (*18*).

Statistical Data Analysis

ANOVA (Student-Newman-Keuls test, $\alpha = 0.05$) and Principal Component Analysis (SPSS version 10.0, SPSS Inc.) were applied to the flavonol profiles data according to the grape cultivar.

Results and Discussion

White Grapes Flavonol Profiles

The flavonol profiles of five representative Spanish white grape cultivars (Airén, Macabeo, Moscatel Grano Menudo, Pedro Ximenez, and Verdejo) and five of the most widespread white grape cultivars in the world (Chardonnay, Gewürztraminer, Riesling, Sauvignon Blanc, and Ugni Blanc) are depicted in Figure 2. Quercetin-type flavonols dominated the flavonol profiles of white grape cultivars, followed by kaempferol-type flavonols. The complete series of the 3-*O*-glucoside, 3-*O*-galactoside and 3-*O*-glucuronide of kaempferol and quercetin were found in all the analyzed samples. With regards to isorhamnetin derivatives, only the 3-*O*-glucoside could be quantified for all the samples, whereas the 3-*O*-galactoside derivative was only quantifiable in a few samples; the 3-*O*-glucuronide derivative was detected by ESI-MS^n, but it could not be quantified. Appreciable differences among the grape cultivars could be observed concerning the relative intensity of the peaks assigned to the 3-*O*-glucoside and the 3-*O*-glucuronide of quercetin, and also among the peaks assigned to the 3-*O*-glucosides of quercetin, kaempferol and isorhamnetin.

The Principal Component (PC) Analysis applied to the profiles of both individual and aglycone-type flavonols allowed some grouping of white grape cultivars (Figure 3). According to PC-1, the grape cultivar Moscatel Grano Menudo had the highest proportions of total and individual kaempferol-type flavonols and the lowest proportion of quercetin-type flavonols, followed by the cultivars Pedro Ximenez, Verdejo and Chardonnay. The grape cultivar Pedro

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Ximenez had the highest proportion of isorhamnetin-type flavonols, namely isorhamnetin 3-*O*-glucoside, followed by the cultivars Gewürztraminer and Verdejo (PC-2, Figure 3a). The rest of the white grape cultivars showed only slight differences in the proportions of the aforementioned flavonols. However, the proportions of the 3-*O*-galactoside and the 3-*O*-glucoside of quercetin allowed some additional differentiation among white grape cultivars (PC-3, Figure 3b): the highest proportions of these two quercetin-type flavonols corresponded to cultivars Chardonnay, Riesling and Ugni Blanc, together with Pedro Ximenez; medium proportions were shown by Airén and Verdejo grapes; Moscatel Grano Menudo and Gewürztraminer showed lower proportions; and the lowest proportions were shown by Macabeo and Sauvignon Blanc.

Red Grapes Flavonol Profiles

The flavonol profiles of five representative Spanish red grape cultivars (Cencibel, Garnacha, Bobal, Monastrell, and Mencía) and five of the most widespread red grape cultivars in the world (Cabernet Sauvignon, Merlot, Pinot Noir, Syrah, and Petit Verdot) were obtained (Figure 4). Quercetin-type and myricetin-type flavonols dominated the flavonol profiles of red grape cultivars. The complete series of the 3-*O*-glucoside, 3-*O*-galactoside, and 3-*O*-glucuronide of the B-ring non-methoxylated flavonol aglycones (kaempferol, quercetin, and myricetin) were found in all the analyzed samples. In addition, the 3-*O*-glucosides of isorhamnetin, laricitrin and syringetin (B-ring methoxylated flavonol aglycones) were detected in quantifiable amounts. The rest of the known red grape flavonols could be detected by means of ESI-MS^n, but they could not be quantified in most cases. The most appreciable differences among the red grape cultivars concerned the relative intensity of the peaks assigned to the 3-*O*-glucoside and the 3-*O*-glucuronide of quercetin, and also within the peaks assigned to the 3-*O*-glucosides of the six flavonol aglycones (kaempferol, quercetin, isorhamnetin, myricetin, laricitrin, and syringetin).

Red grapes contain a higher number of individual flavonols in comparison to white grapes. Therefore, the Principal Component (PC) Analysis applied to red grape flavonol profiles (both individual and aglycone-type flavonol profiles) allowed a high degree of grape cultivar differentiation (Figure 5). The PC-1 was more correlated to the molar proportions of the main flavonols (myricetin-type, especially myricetin 3-*O*-glucoside, and quercetin-type). Pinot Noir grapes had the highest proportion of quercetin-type flavonols and the lowest proportion of myricetin-type flavonols, followed by the cultivars Garnacha (Grenache) and Monastrell. The grape cultivars Bobal, Mencía, Cabernet Sauvignon and Merlot had medium proportions of quercetin-type and myricetin-type flavonols. On the other hand, the grape cultivars Cencibel (Tempranillo), Syrah and Petit Verdot showed the lowest proportions of quercetin-type flavonols and the highest proportions of myricetin-type flavonols. The recently reported laricitrin-type and syringetin-type flavonols offered interesting cultivar differentiation along the PC-2 (Figure 5a): Petit Verdot grapes had higher proportion of these two types of flavonols than Cencibel and Syrah cultivars and the same was found in the cases of Cabernet Sauvignon and Mencía in comparison to Bobal and

Merlot and in Monastrell when compared to Garnacha. Finally, the proportions of isorhamnetin-type flavonols (especially the 3-*O*-glucoside) and myricetin 3-*O*-glucuronide (PC-3, Figure 5b) allowed an additional differentiation between the pairs of grape cultivars Cencibel and Syrah, and Mencía and Cabernet Sauvignon, and reinforced the differentiation between Monastrell and Garnacha. The cultivars Syrah and Garnacha had the lowest proportions of myricetin 3-*O*-glucuronide and the highest proportions of isorhamnetin-type flavonols.

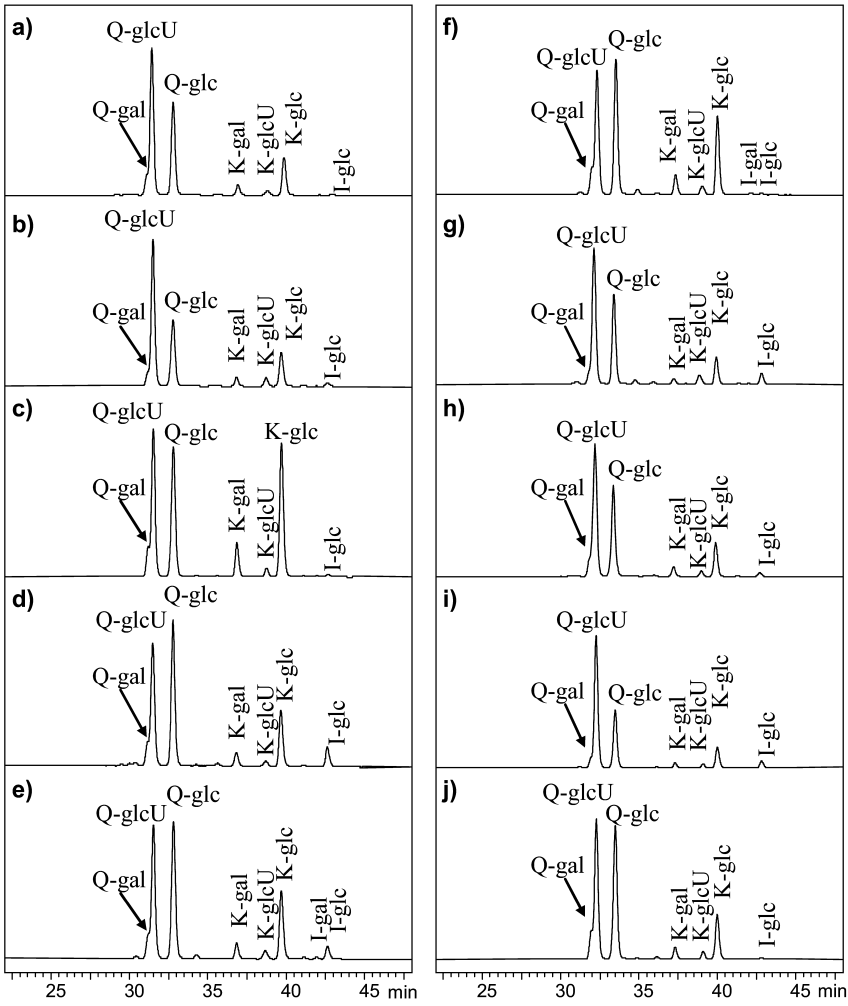

Figure 2. Characteristic flavonol profiles (HPLC chromatograms at 360 nm) of the following white grape cultivars: a) Airén; b) Macabeo; c) Moscatel Grano Menudo; d) Pedro Ximenez; e) Verdejo; f) Chardonnay; g) Gewürztraminer; h) Riesling; i) Sauvignon Blanc; j) Ugni Blanc. Abbreviations: K, kaempferol; Q, quercetin; I, isorhamnetin; glc, 3-O-glucoside; gal, 3-O-galactoside; glcU, 3-O-glucuronide.

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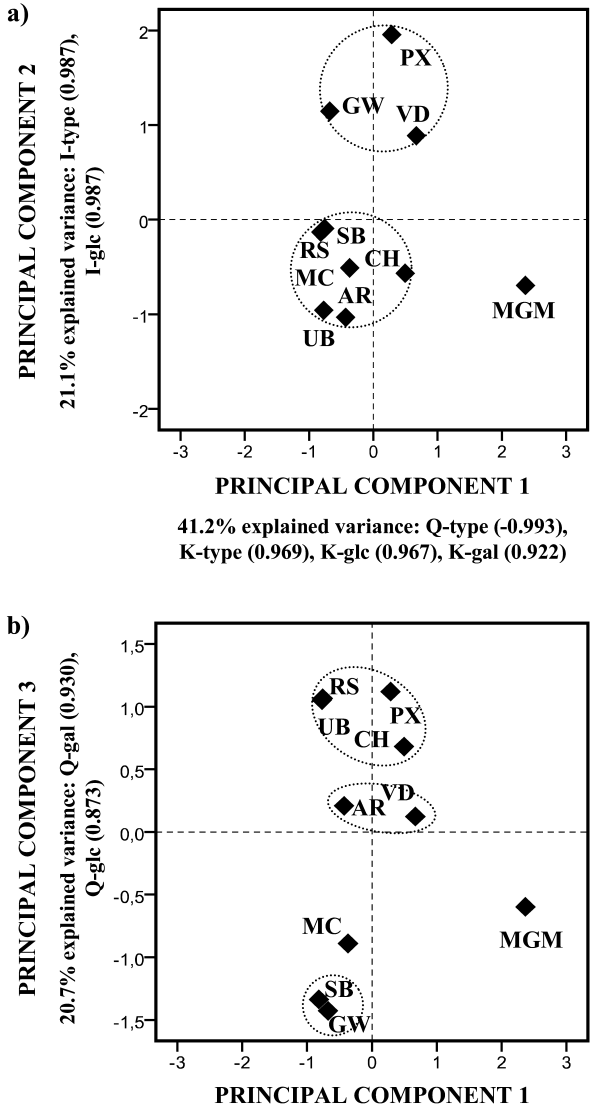

Figure 3. Plot of white grape samples in the space determined by Principal Component (PC) Analysis: a) PC-1 vs. PC-2; b) PC-1 vs. PC-3. White grape cultivars: AR, Airén; MC, Macabeo; MGM, Moscatel Grano Menudo; PX, Pedro Ximenez; VD, Verdejo; CH, Chardonnay; GW, Gewürztraminer; RS, Riesling; SB, Sauvignon Blanc; UB, Ugni Blanc.

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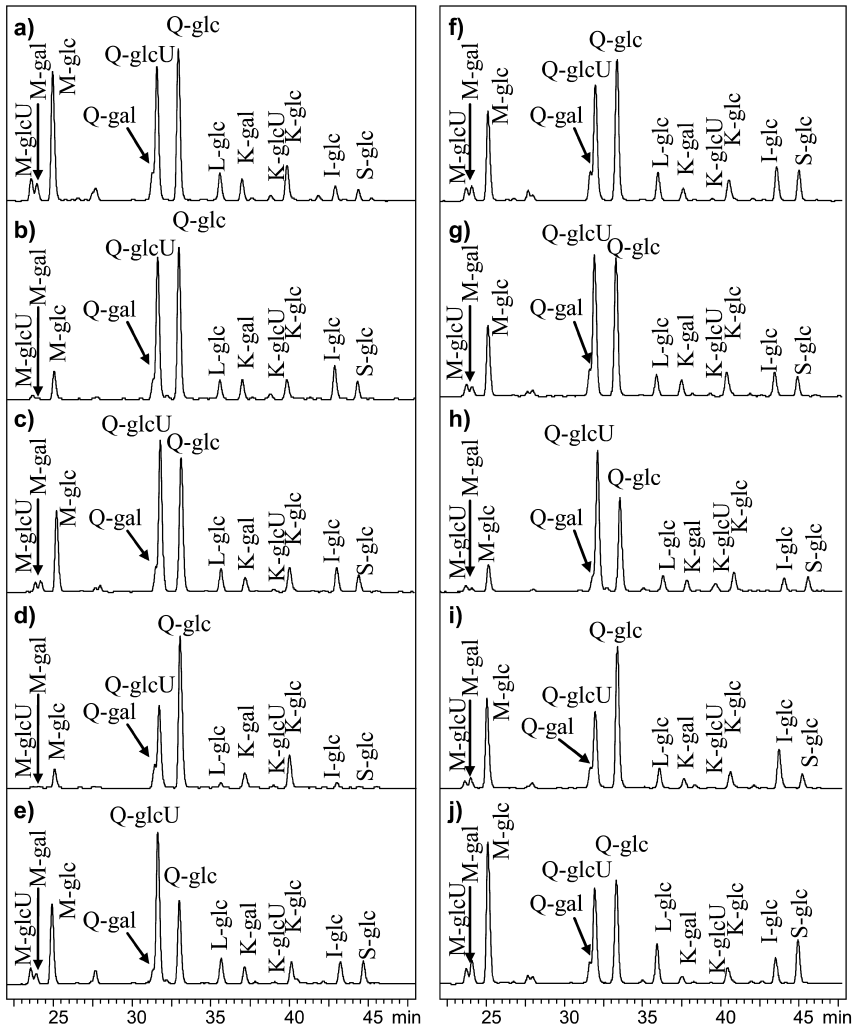

Figure 4. Characteristic flavonol profiles (HPLC chromatograms at 360 nm) of the following red grape cultivars: a) Cencibel (Tempranillo); b) Garnacha (Grenache); c) Bobal; d) Monastrell; e) Mencía; f) Cabernet Sauvignon; g) Merlot; h) Pinot Noir; i) Syrah; j) Petit Verdot. Abbreviations: K, kaempferol; Q, quercetin; I, isorhamnetin; M, myricetin; L, laricitrin; S, syringetin; glc, 3-O-glucoside; gal, 3-O-galactoside; glcU, 3-O-glucuronide.

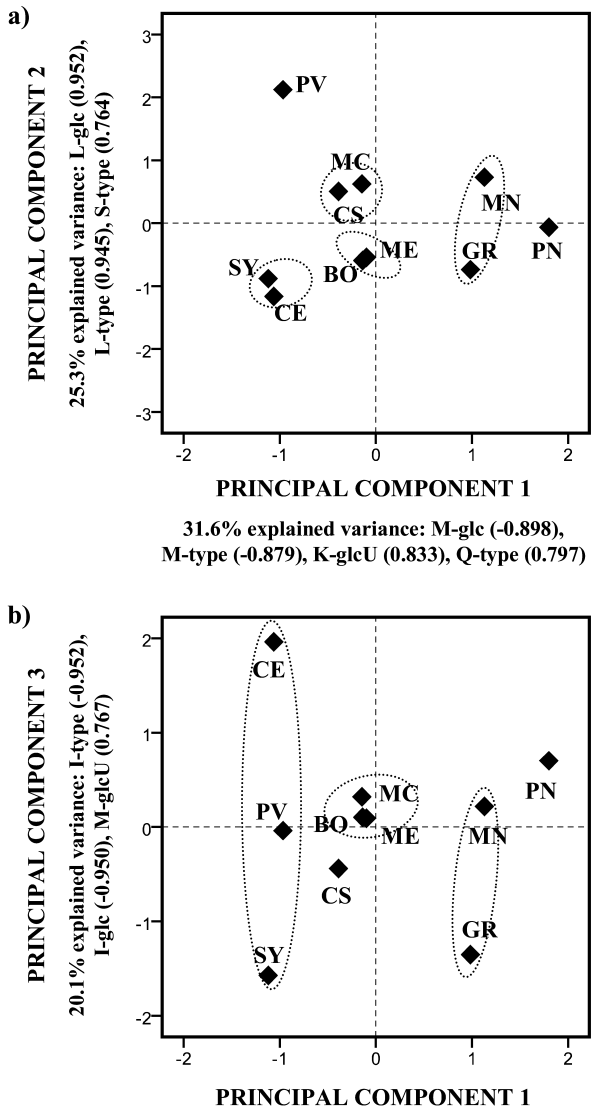

Figure 5. Plot of red grape samples in the space determined by Principal Component (PC) Analysis: a) PC-1 vs. PC-2; b) PC-1 vs. PC-3. Red grape cultivars: CE, Cencibel (Tempranillo); GR, Garnacha (Grenache); BO, Bobal; MN, Monastrell; MC, Mencía; CS, Cabernet Sauvignon; ME, Merlot; PN, Pinot Noir; SY, Syrah; PV, Petit Verdot.

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Red Wines Flavonol Profiles

Wine flavonol analysis by the HPLC method developed by our group can be performed by direct injection of wines. This is true for white wines and it is applicable to most red wines. Red wine anthocyanins cause some interference, especially in young deep-red wines, but the main flavonols are not affected. Clearer chromatograms can be obtained by the previous isolation of the free-anthocyanin flavonol fraction of red wine following the procedure described for red grape skins extracts (see Experimental section). However, some losses of free flavonol aglycones were observed when free-anthocyanin flavonol fractions of red wine samples were isolated, whereas the flavonol 3-*O*-glycosides remained intact. We recommend the direct injection of wine samples as it is cheaper and less time-consuming, offering good results.

The use of flavonol profiles in wine for cultivar authentication and differentiation purposes is handicapped by the partial hydrolysis suffered by the flavonol 3-*O*-glycosides. Therefore, wines show a complex mixture of flavonol 3-*O*-glycosides together with the flavonol aglycones released from them. This situation is well illustrated in Figure 6, showing three red wines of the same grape cultivar with different degrees of flavonol hydrolysis (Figures 6b to 6d) in comparison to the original flavonol 3-*O*-glycosides present in the skins of their corresponding grape cultivar (Figure 6a). The 3-*O*-glucuronide derivatives and the syringetin-type flavonols seem to be the most resistant to hydrolysis (Figure 6d). Moreover, red wines with low degrees of flavonol hydrolysis showed flavonol profiles that differed from those of their corresponding grape flavonol profiles. A comparison between Figures 6a (Cencibel grape) and 6b (Cencibel wine) shows a relative increase of myricetin-type flavonols and a parallel decrease of quercetin-type flavonols in red wines. Similar differences have also been described for anthocyanin profiles of red grape and wine (*24–26*).

The flavonol profiles of 150 single-cultivar red wine samples (vintages 2001 to 2007) of 10 representative Spanish (Cencibel, Tempranillo, Bobal, Garnacha, Mencía, and Monastrell) and worldwide spread (Cabernet Sauvignon, Merlot, Petit Verdot, and Syrah) grape cultivars were obtained. In addition, we analyzed 28 wine samples of a common Spanish blend of single-cultivar wines (Tempranillo and Cabernet Sauvignon in unspecified proportions). The direct statistical analysis (ANOVA and Principal Component Analysis) of the flavonol profile data of the aforementioned red wine samples did not lead to clear results because of the very diverse hydrolysis degree shown by the wines. Moreover, the hydrolysis did not affect the different flavonol 3-*O*-glycosides to the same extent and the degree of hydrolysis did not seem to correlate to wine age.

For the above mentioned reason, we decided to express the results of the red wine flavonol profiles on an aglycone-type basis, by the total sum of the molar percentages of all flavonol 3-*O*-glycosides having the same kind of flavonol aglycone (e.g., the 3-*O*-glucoside, the 3-*O*-galactoside and the 3-*O*-glucuronide of quercetin) and their own free flavonol aglycone (e.g., quercetin). The resulting aglycone-type flavonol profiles are shown in the Table, together with the total content (μmol/L) of flavonols, according to the grape cultivar used for the elaboration of wines.

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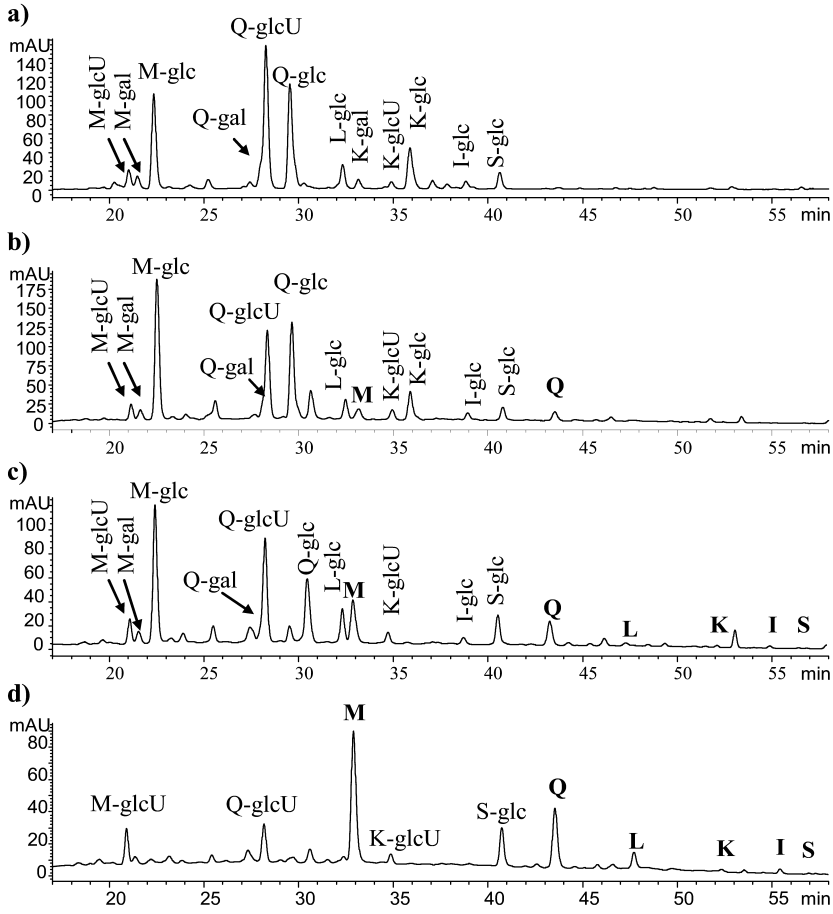

Figure 6. Comparison of the flavonol profiles (HPLC chromatograms at 360 nm) of: a) Cencibel (Tempranillo) grape skin extract showing only flavonol 3-O-glycosides; b-d) Cencibel (Tempranillo) wines showing different hydrolysis degrees of flavonol 3-O-glycosides. Abbreviations: K, kaempferol; Q, quercetin; I, isorhamnetin; M, myricetin; L, laricitrin; S, syringetin; glc, 3-O-glucoside; gal, 3-O-galactoside; glcU, 3-O-glucuronide.

Table 1. Mean values and standard deviations (MV ± SD of the aglycone-type flavonol profiles (molar percentages) and total flavonol content (µmol/L) of commercial red wines

Wine Samples	% K-type	% Q-type	% I-type	% M-type	% L-type	% S-type	Total flavonols
Cencibel (n = 28)	3.6 ± 1.1 bc	35.4 ± 2.6 bc	2.6 ± 0.6 a	46.5 ± 2.0 d	7.0 ± 1.0 b	4.9 ± 1.1 a	207 ± 60 b
Tempranillo (n = 49)	3.4 ± 0.7 bc	35.6 ± 2.3 bc	2.4 ± 0.5 a	46.1 ± 2.6 d	7.3 ± 0.8 b	5.2 ± 1.0 a	189 ± 45 b
Bobal (n = 4)	2.5 ± 0.8 abc	39.6 ± 5.9 cd	3.1 ± 0.4 ab	39.5 ± 4.1 bc	7.1 ± 1.7 b	8.1 ± 2.4 c	161 ± 56 b
Garnacha (n = 9)	3.5 ± 1.2 bc	43.0 ± 6.0 de	4.2 ± 1.2 b	36.6 ± 6.1 abc	6.3 ± 1.0 ab	6.3 ± 1.9 abc	147 ± 61 b
Mencía (n = 4)	3.4 ± 1.0 bc	20.0 ± 1.9 a	3.7 ± 2.1 ab	50.6 ± 4.5 e	8.9 ± 1.3 c	13.4 ± 2.5 d	81 ± 48 a
Monastrell (n = 6)	3.1 ± 0.9 bc	46.6 ± 3.5 e	2.9 ± 1.4 ab	37.0 ± 1.1 abc	5.5 ± 1.0 a	4.8 ± 1.4 a	175 ± 42 b
Cabernet Sauvignon (n = 14)	3.4 ± 1.0 bc	43.0 ± 3.7 de	3.9 ± 1.0 b	35.5 ± 4.6 ab	6.8 ± 1.1 ab	7.4 ± 1.6 bc	174 ± 56 b
Merlot (n = 13)	3.2 ± 0.9 bc	45.7 ± 4.9 e	4.2 ± 1.2 b	33.7 ± 4.3 a	6.8 ± 1.4 ab	6.4 ± 2.0 abc	178 ± 56 b
Petit Verdot (n = 4)	1.5 ± 0.6 a	34.4 ± 4.3 b	2.5 ± 1.0 a	33.3 ± 3.7 a	12.1 ± 2.7 d	16.2 ± 2.0 e	153 ± 28 b
Syrah (n = 19)	2.3 ± 0.7 ab	38.5 ± 2.8 bc	7.4 ± 1.2 c	35.2 ± 2.2 a	9.0 ± 0.9 c	7.5 ± 1.2 bc	274 ± 77 c
Tempranillo + Cabernet Sauvignon (n = 28)	3.8 ± 0.8 c	39.5 ± 2.7 cd	3.1 ± 0.9 ab	40.5 ± 3.0 c	7.1 ± 0.8 b	5.9 ± 1.3 ab	154 ± 30 b

[abcde] Different letters in the same column means significant differences according to ANOVA (Student-Newman-Keuls test, $\alpha = 0.05$). K, kaempferol; Q, quercetin; I, isorhamnetin; M, myricetin; L, laricitrin; S, syringetin

The main flavonols found in red wine were myricetin-type (mean value ranging between 33.3 and 50.6%) and quercetin-type (mean value ranging between 20.0 and 46.6%), as observed for red grapes. The recently reported laricitrin-type and syringetin-type flavonols usually accounted for molar percentages higher than 5% (5.5-12.1% for laricitrin-type, and 4.8-16.2% for syringetin-type), whereas isorhamnetin-type and kaempferol-type flavonol were minor compounds (less than 5%, with the exception of Syrah wines containing 7.4% of isorhamnetin-type flavonols). Ampelographic and AFLP molecular markers have demonstrated that Tempranillo and Cencibel are two synonymous names of the same grape cultivar (*27*). Consequently, it was not surprising that the aglycone-type flavonol profiles of the analyzed Cencibel and Tempranillo wines were statistically indistinguishable, although some Spanish winemakers use both names as they corresponded to different cultivars. The total flavonol content of a red wine can be affected by many factors, some of which are related to sun exposure of grape cluster (*1–3*) and, very likely, other factors which are related to winemaking practices. In fact, the total flavonol content found in the analyzed red wine samples ranged between 39 and 473 μmol/L, and wide and overlapping ranges were observed for grouped single-cultivars wines (Table, mean values between 147 and 207 μmol/L). However, two single-cultivar wines showed characteristic total flavonol contents: the lowest content was found in Mencía wines (mean value, 81 μmol/L), whereas the highest content corresponded to Syrah wines (mean value, 274 μmol/L).

The Principal Component (PC) Analysis of the aglycone-type flavonol profiles of single-cultivar red wines showed that laricitrin-type and syringetin-type flavonols contributed to a greater extension in wine differentiation (Figure 7, PC-1) than the main flavonols (myricetin-type and quercetin-type; Figure 7, PC-2). The single-cultivar red wines were grouped, following a decreasing order of the proportions of laricitrin-type and syringetin-type flavonols, in: Petit Verdot – Mencía and Syrah – the rest of the wines. According to PC-2, Mencía, Cencibel and Tempranillo wines had the highest proportions of myricetin-type flavonols and the lowest proportions of quercetin-type flavonols. Cabernet Sauvignon and Merlot wines showed quite similar aglycone-type flavonol profiles, showing the highest proportions of quercetin-type flavonols and the lowest proportions of myricetin-type flavonols. Tempranillo-Cabernet Sauvignon blended wines showed an intermediate aglycone-type flavonol profile and the plot of these samples overlapped with the plot areas occupied by Cencibel/Tempranillo and Cabernet Sauvignon wine in Figure 7 (data not shown). Garnacha and Monastrell wines showed aglycone-type flavonol profiles quite similar to those of Cabernet Sauvignon and Merlot wines. However, wines of these varieties can easily be differentiated by their anthocyanin profiles (*17*, *26*). Finally, Bobal wines did not show a very characteristic aglycone-type flavonol profile and the few represented samples overlapped with the other groups.

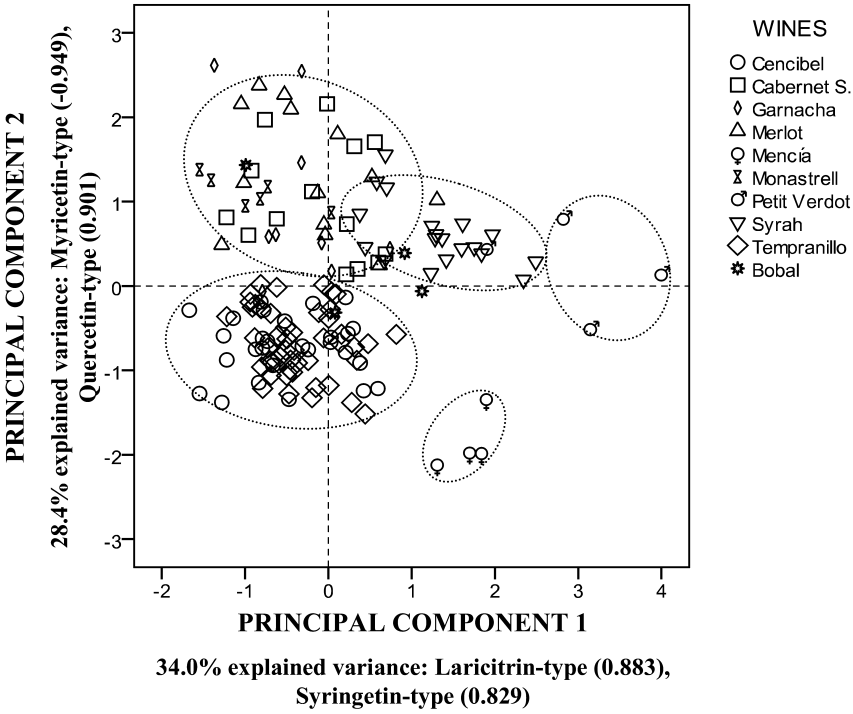

Figure 7. Plot of red wine samples in the space determined by Principal Component (PC) Analysis: PC-1 vs. PC-2.

Conclusions

Flavonol profiles can be considered chemical markers for grape and wine cultivar authentication and differentiation. However, the flavonol profile of a single-cultivar wine differs fron that of its corresponding grape cultivar. On the one hand, the transfer of flavonol 3-*O*-glycosides from grape to wine during winemaking does not seem to be equal for all kinds of flavonols. On the other hand, flavonol 3-*O*-glycosides suffer partial hydrolysis in wine. For the latter reason, wine flavonol profiles need to be expressed on a basis of aglycone-type flavonols in order to compensate the changes introduced by flavonol hydrolysis.

In the case of white grapes, the major quercetin-type flavonols, followed by the minor isorhamnetin-type flavonols, permitted cultivar authentication and differentiation. Similarly, for red grapes the major quercetin-type and myricetin-type flavonols, followed by the minor laricitrin-type and syringetin-type flavonols, allowed us cultivar authentication and differentiation. In contrast, the minor laricitrin-type and syringetin-type flavonols were more important than the major myricetin-type and quercetin-type in cultivar authentication and differentiation of red wines.

Acknowledgments

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Chapter 9

Authentication of Different Terroirs of German Riesling Applying Sensory and Flavor Analysis

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The term "terroir", which originated in France, comprises the interaction of soil, climate, and topography with the vines of a specific variety. It may be extended to the human impact by viticultural and oenological measures. In order to study the sensory impact of terroir, 25 highly diverse vineyard sites from the growing regions Ahr, Mosel, Nahe, Pfalz and Rheinhessen were selected. For the vintages 2004, 2005, 2006 and 2007 sound Riesling grapes were harvested from those sites at optimal maturity. Grapes from each vineyard site were split in two, with one part subjected to a standardized winemaking protocol, and the other portion undergoing customary winemaking at the respective cooperating wine estates. Eight months after harvest, a descriptive analysis by up to 20 trained judges characterized the wines by one color, 14 odor and five taste attributes. Wines coming from different vineyard sites yielded considerable variation. For example, wine made from Riesling grapes grown on a loamy loess soil with basalt stones was much more intensive in its citrus, peach, mango and cantaloupe attributes than the wine produced from vines growing in light colored sandstone, which was described as more sour with vegetative and mineral notes. Applying discriminant analysis, it was possible to group the wines from the five bedrock types (sandstone, loam loess, rotliegend, slate and limestone)

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according to their sensory properties, and identify those aroma notes that were typical for each type of bedrock.

Keywords: sensory evaluation; Riesling; terroir; aroma compounds; volatiles

In contrast to many other foods or beverages, many wines are not marketed as commodities, but as regional products made from specific grape varieties. Thus, labels refer to numerous entities such as geographic origin, grape variety, vintage, national quality hierarchies and even winemaking techniques, such as aging in oak barrels. Due to the multitude of legal specifications and the enormous price range in which wines are offered, the authentication of wine is a highly complex and ambitious endeavor. Among wine experts but also educated consumers world wide, a paradigm exists which claims that the smaller the geographic area/site from which the grapes for a specific wine are sourced, the higher the quality designation and very often the price tag of the subsequent wine. In light of authentication, two very important questions arise: Does the specific configuration of natural factors, which defines a distinctive geographic origin, increasingly referred to as "terroir", cause a sufficient impact on the chemical composition of finished wines, so that wine experts and even consumers are indeed able to discriminate them by sensory means and actually recognize similar types of bedrock such as lime stone, slate or sandstone? If this is true, the next question would address the role and importance of individual climatic and topographic factors defining specific vineyard sites or terroirs on sensory properties and chemical wine composition, especially volatiles.

To perform successfully in national wine markets under the circumstances of growing competition, fueled by the ongoing globalization in wine trade, wines need a unique selling proposition (USP) which simultaneously attracts consumers' interest. Terroir, the specific combination of a vineyard's soil composition, meso-climate and topography in interaction with the vines has been widely acknowledged as an important factor in wine quality and style (*1*) . These site specific factors represent physically immovable properties, which are unique for local wine growing areas and cannot be imitated elsewhere, such as grape varieties, winemaking technologies or yeast strains. Thus, terroir may serve as an excellent USP, and indeed, not only traditional quality hierarchies such as the distinction of village, premier cru or grand cru wines in Burgundy, France, rely on the terroir concept, but also more recent marketing strategies, which try to emphasize small vineyard sites or even the type of bedrock in a particular vineyard. Indeed, consumers are strongly interested in how different terroirs translate into particular sensory properties, and how they can acquire an expertise in appreciating and even distinguishing different terroirs based on the sensory appearance of the wines.

Numerous studies succeeded in differentiating between wines according to their geographic origin, based on the wines' sensory properties (*2–8*) and vintage (*9–12*). Sensory analysis of 24 commercial Chardonnay wines from the Niagara Peninsula sub-appellations in Canada, plus three non-Canadian Chardonnays,

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detected subtle regional variations via principal component analysis (PCA), although it failed to classify the samples by origin (*13*). This could be a result of the vinification process, which possibly masked the site-specific aromatic character by oenological treatments such as malolactic fermentation or aging in oak barrels. For 14 Riesling wines from the same sub-appellations however, sensory analysis provided a distinct classification via PCA and discriminant analysis (DA) (*2*). It could be speculated, that Riesling as a variety exhibits a stronger tendency to show terroir differences than Chardonnay, or that the winemaking applied for Riesling wines itself has a less dominant sensory impact than the common fermentation and aging in oak barrels for Chardonnay wines.

Examining the sensory profiles of commercial Riesling wines from two consecutive vintages, five wine estates and six vineyard designations in the Rheingau region in Germany, the effects of vintage, terroir and individual vinification applied by different wine estates were investigated. The substantial sensory heterogeneity within identical vineyard designations led to the conclusion that the impact of vintage and individual viticultural and winemaking practices of each estate dominated over the impact of geographic origin (*14*). However, this experiment was flawed by the fact that wines of both vintages were evaluated at the same time and wines of the older vintage were more aged. Hence, the effect of vintage was artificially enhanced by the additional year of maturity. Several Spanish studies evidenced correlations between wines' sensory properties as well as their volatiles composition and their geographic origin (*8*, *15–17*). Especially volatile compounds generated from per-oxidation of unsaturated fatty acids and the yeast amino acid metabolism seemed to be most suitable for differentiating between geographic origins in Spanish rose and red wines (*15*). With five Chianti wines from different production areas in Tuscany, region-specific differences among volatiles were observed, particularly regarding the contents of ethyl octanoate, γ-butyrolactone and ethyl hexanoate (*18*). Applying discriminant analysis and artificial neural networks on aroma chemical data, 59 Austrian white wines of the variety Grüner Veltliner were successfully classified by origin with high accuracy (*19*). Numerous studies with French wines also evidenced the influence of geographic origin on the aroma profile and composition of wines (*4*, *20*, *21*): ANOVA on sensory data of 30 red wines from the regions of Bordeaux, Burgundy, Beaujolais and Rhône revealed significant regional differences in 15 of 17 attributes. PCA discriminated these wines by geographical origin. While classification based on chemical parameters reached 81.8 % accuracy, classification using exclusively sensory data gave only 63.3 % accuracy (*21*). Although there are many detailed descriptions on geological and climatic characteristics of viticultural areas, and ample studies succeeding in differentiating between growing regions (*22*, *23*), grape varieties or vintages by means of sensory or aroma analysis, there is still a scientific gap regarding a conclusive sensory explanation of the natural growing conditions. In order to go beyond the mere description of terroir diversity, this research will systematically address the sensory and chemical impact of a number of bedrock and soil types at different locations and across consecutive vintages. Furthermore, statistical analysis will be applied to elucidate correlations between terroir-specific climate

or soil data and sensory properties of the wines produced from these sites, as well as the site-specific generation of aroma compounds in the finished wines.

Experimental

Sensory Analysis

Twelve highly diverse vineyard sites from ten wine estates were selected within the German wine growing region Pfalz, which covers 5500 ha Riesling acreage (*24*) and provides 9% of the world's Riesling plantings (*25*). The substrates yielding the soils of these sites included limestone, sandstone, greywacke, basalt and breccia from the rotliegend age (Lower Permian). After one year, the research was extended to 13 additional sites in the German growing regions Mosel, Ahr, Nahe and Rheinhessen. These vineyards comprised further sites on limestone and rotliegend material, as well as sites on slate and porphyry. All Riesling vineyards were at least ten years old, and besides a few sites at the Mosel, all vineyards were trained by vertical shoot position. Riesling clones varied substantially (Gm239, Gm198, N90, 21B, Weis 21, 356 Fin, BKS68), while the majority of vines were grafted on rootstock SO4, followed by 5C, 26G and 125AA. In the vintages 2004, 2005, 2006 and 2007 sound Riesling grapes were harvested from these sites at optimal maturity, which was individually determined by the cooperating wine estates. One portion of grapes from each site was subjected to a standardized winemaking protocol, while the major portion underwent customary vinification at the respective wine estates. This division additionally allowed for evaluating the impact of individual winemaking on the wines' sensory properties, except for the choice of yeast, as all wines were fermented with the same commercial yeast strain (Lalvin R-HST, Lallemand, Rexdale, Canada).

Eight months after harvest, 20 trained judges characterized the wines of the 2004 vintage by color intensity, 14 odors (mineral, lemon/grapefruit, rhubarb, apple, peach, mango/passion fruit, cantaloupe, honey/caramel, smoky, floral, green grass/cucumber, box tree, green bean, buttery/bread crust/yeasty/sweaty) and five taste attributes (sweet, sour, harsh acidity, hard mouth feel, bitter) in a descriptive analysis. For the 2005, 2006, and 2007 vintages, which were also assessed eight month after each harvest, two tasting panels were employed, consisting of 13 and 15 trained judges respectively. The array of taste descriptors was extended by the attribute minerality, while the odor attribute green bean was eliminated. The wines from the standardized vinification were sampled in triplicate, while those from the wine estates were evaluated in duplicate.

Sensory assessment of the 2004 wines was repeated with the same trained panel after four years in 2008, using the same array of sensory descriptors as in the 2005 vintage.

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Aroma Analysis

A volume of 14 ml undiluted wine was filled into 20 ml glass vials, supplemented with 4.2 g NaCl and a glass covered magnetic stir bar and closed by a Teflon-coated septum. For FID-detection 35 µg 2-Heptanol was added as the internal standard, and 0.14 µg of ethyl-(methylthio) acetate for PFPD-detection. Solid-phase-micro-extraction (SPME) was performed by using a 50/30 µm divinylbenzene/carboxen/polydimethlysiloxan fiber (Supelco Inc., Bellefonte(PA), USA). Extraction of volatiles in the headspace lasted 90 min with the sample being constantly agitated at 35°C, using a CombiPAL autosampler (CTC Analytics, Switzerland) equipped with a special oven. The loaded fiber was desorbed in the injector at 220°C for 5 min. When the fiber was removed, the injection mode switched from split less to a 1:10 split ratio. Gas chromatographic analysis was performed with a Varian CP 3800 GC (Varian Inc., Palo Alto (CA), USA) and a ZB-Wax capillary column (30 m x 0.25 i.d. x 0.25 µm thickness) (Phenomenex, Aschaffenburg, Germany) operated at a constant flow rate of 1.2 ml min^{-1} H_2. The temperature program started at 40°C, which was maintained for 2 min, followed by an increase at 4°C min^{-1} up to 240°C, which was kept constant for 10 min. At the end of the column the effluent was split by 1:1 and either directed towards a flame-ionization-detector (FID) operated at 250°C with N_2 at 25 ml min^{-1}, H_2 at 28 ml min^{-1} and purified air at 300 ml min^{-1}, or towards a pulsed-flame-photometric-detector (PFPD) for selective analysis of sulfurous compounds, operated at 250°C with H_2 at 11 ml min^{-1} and purified air at 17 ml min^{-1}.

All wine samples were re-analyzed on a ZB-5 capillary column of different polarity (30 m x 0.25 i.d. x 0.5 µm thickness) (Phenomenex, Aschaffenburg, Germany). For this purpose, the H_2 flow rate was raised to 2 ml min^{-1} and the modified temperature program started at 35°C, which was kept constant for 5 min, followed by a rise at 3°C min^{-1} up to 200°C, from which the temperature increased again after 1 min rest at a rate of 8°C min^{-1} to the final level of 300°C, which was maintained for 5 min.

A sub-set of four wines of each vintage was also analyzed by GC mass spectrometry, using the previously described extraction and injection procedure (however at an injection temperature of 230°C), and performed by a CombiPAL autosampler (CTC Analytics, Switzerland). The Trace GC 2000 / DSQ GC-qMS combination (Thermo Electron, Dreieich, Germany) was equipped with a ZB-Wax column (see above). At a flow rate of 1.2 ml He min^{-1}, the temperature program was initially held constant for 5 min at 35°C, then increased at 4°C min^{-1} up to 240°C and kept constant for an additional 10 min. Fragmentation by electronic ionization at 70 eV took place at 240°C. Data acquisition in total ion mode covered a mass range of 29 to 250 atom mass units (amu) for the first 40 min, and 29 to 450 amu for the final 20 min. The identification of unknown substances was based on retention indices on the polar (ZB-Wax) and medium polar (ZB-5) columns, as well as MS spectra matched to the NIST database.

Results and Discussion

Impact of Individual Geological Substrates

Confining terroir to the substrates that soils have derived from, is a gross simplification from a scientific perspective, considering the great number of pedeologic, topographic and climatic factors which impact the development of vines, and therefore also significantly contribute to the sensory properties of wines grown on specific sites. However, this simplification is now increasingly used in wine marketing, as winemakers progressively replace the name of vineyard sites on bottle labels or price lists with the types of geological substrate or bedrock found in these sites, such as slate, sandstone or limestone. Consequently, consumers expect that geological substrates do have an impact on the sensory properties of wines. This demands the examination of to what extent this consumer expectations can be confirmed by sensory analysis.

Indeed, comparing the sensory profiles of two terroirs in Figure 1, which are only two km apart from each other and cultivated by the same wine estate, the two types of bedrock had a highly significant impact on nine odor and four taste attributes. The bedrock type basalt with its higher loam content yielded a Riesling with stronger fruit character being traditionally associated with German Riesling (lemon/grapefruit, apple, peach, rhubarb), as well as notes of exotic fruits (mango/passion fruit, cantaloupe), which are presumably related to higher thiol contents. The sandstone-derived soil of the Deidesheimer Kieselberg, which is lighter and contains a substantial portion of gravel, produced a Riesling with lower fruit intensities, more vegetative aromas and a stronger acidity which was perceived as being harsher and which led to a hard mouth feel.

In contrast, Figure 2 shows a comparison of two wines which were produced from the same type of bedrock (rotliegend, which comprises reddish breccias or iron-rich slate material) but which came from two vineyard sites, being 150 km apart from each other and hence substantially different regarding their meso-climate (Birkweiler, Pfalz and Ürzig, Mosel). Both wines displayed a surprisingly high degree of similarity: Only two odor attributes (rhubarb and apple) differed noticeably in their intensities. Among the taste attributes, only bitterness, harsh acidity and hard mouth feel showed significant differences. This may be attributable to the higher alcohol content (+2% vol.) of the wine from the Pfalz, as ethanol is known to enhance bitterness (*26*).

In summary, two vineyards that differed in their geological substrate produced two very distinctive wines, although these vineyards are located in close proximity, leading to a nearly identical meso-climate, and despite the equal viticultural regime applied by the same wine estate. At the same time, wines from two vineyards with the identical type of bedrock, however in great distance from each other and with considerable meso-climatic diversity (Pfalz versus Mosel), showed very similar sensory profiles. Bearing these comparisons in mind, it seems reasonable to conclude that a vineyard's geological substrate or type of bedrock has a significant impact on the sensory properties of Riesling wines produced there.

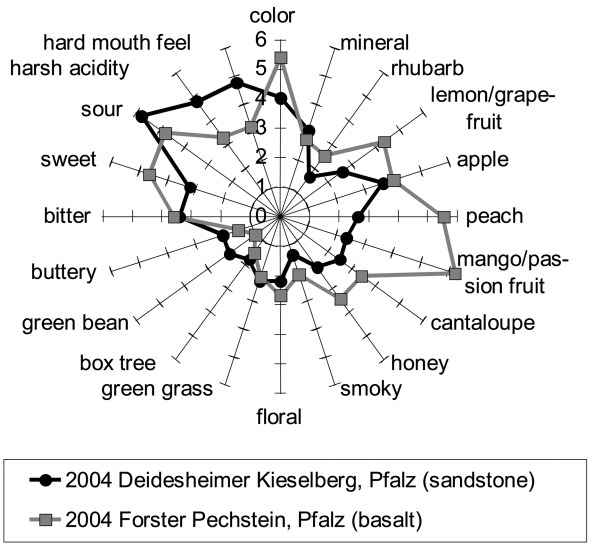

Figure 1. Sensory profiles of the bedrock types sandstone (Kieselberg) and basalt (Pechstein), both vinified at the Bassermann-Jordan estate (n = 20 jdgs. x 2 reps.)

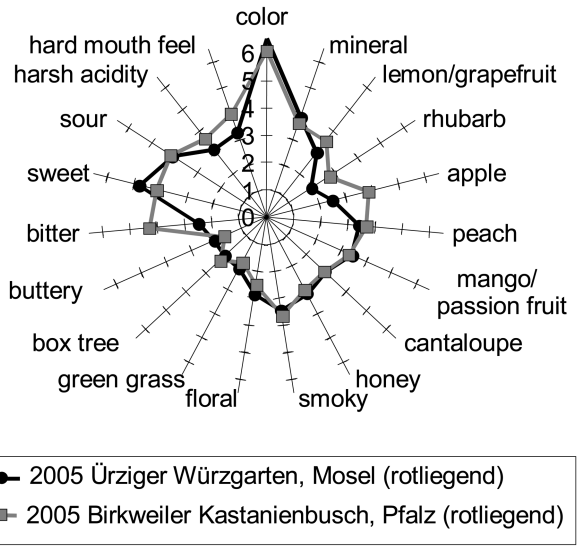

Figure 2. Sensory profiles of Riesling from two Rotliegend terroirs, vinified at the experimental cellar (n = 13 jdgs. x 3 rep.)

Applying an analysis of variance (ANOVA) to all wines of the 2004 vintage (data not shown, refer to (*27*)), the type of geological substrate proved to have a larger impact on the sensory properties of the wines than vinification : 12 out of 20 attributes differed significantly between the substrates, including the

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fruity notes with the exception of "lemon/grapefruit", the vegetative notes with the exception of "green grass/cucumber", and all the taste descriptors with the exception of "bitter". In the 2005 vintage, however, only four taste attributes showed a significant variation between the substrates, presumably due to the atypically dry and hot weather conditions during the ripening period. However, using discriminant analysis (DA) it was still possible for both vintages to clearly distinguish between the different kinds of substrate according to the wines' sensory properties. Based on PCA and DA, typical aroma descriptors could be assigned to each type of bedrock. The wines from basalt were perceived as markedly fruity-aromatic with high intensities in "cantaloupe", "peach/apricot", "mango/passion fruit", "lemon/grapefruit" and "smoky". The wines from limestone also expressed an intensive fruity-sweet aroma, particularly regarding "mango/passion fruit" and "peach/apricot". They displayed a well-balanced acidity on the palate, combined with a smooth mouth feel. The wines from sandstone, as well as those from greywacke, could be recognized through their distinctive, harsh acidity and hard mouth feel. These only had subtle fruity-sweet aromas, but showed a marked vegetative character. In contrast, the wines from the rotliegend substrate showed high intensities in "honey/caramel", "cantaloupe", "rhubarb", "peach/apricot" and "mango/passion fruit" odors, and on the palate expressed a well-balanced acidity and a smooth mouth feel. The wines from slate were characterized by acidity, and "apple", "citrus" and vegetative notes. There was only one wine from porphyry, which also had a distinctive acidity with higher intensities in the odors "mineral", "lemon/grapefruit", "apple" and "peach/apricot". However, individual wines differed from these sensory characterizations, particularly in 2005.

Impact of Vintage and Maturation

Figure 3 shows a comparison of sensory properties of Riesling wines produced from the vineyard sites Deidesheimer Kieselberg (sandstone) and Forster Pechstein (basalt) through the vintages 2004 – 2007. In the score plot of the PCA, both terroirs were grouped together well, despite substantial variation among vintages. The sandstone terroir of the Kieselberg varies less between vintages. It is defined by a pronounced acidity which is perceived as being sharp, creating a hard mouth feel. The bouquet is dominated by vegetative odor attributes (green grass, box tree), whereas fruit attributes are less intense, except for apple and citrus/grapefruit in vintage 2005. The basalt terroir of the Pechstein varies more between vintages, but only the score of the 2005 wine was in proximity to the scores of the Kieselberg wines. While the 2004 and 2005 wines displayed a higher acidity combined with intense apple, citrus/grapefruit odors, the 2006 and 2007 wines were less sour and more floral.

The largest sensory variation was observed for the 2004 vintage, displayed by the longest distance between both vineyard scores, while vintage 2005 yielded the smallest variation, and 2006 and 2007 showed intermediate distances between their scores. It could be speculated that the longer and cooler the vegetation period and the later the harvest, the more sensory variation between terroirs is present. A warm summer and fall however, seems to either hinder sufficient aroma synthesis

within the berries for more sensory discrimination, or foster the wine estates to early picking due to an early sugar accumulation. Harvesting too late in a warm vintage like 2005 would translate into alcohol contents above 13.5 % v/v alc., which are not typical for the desired Riesling style.

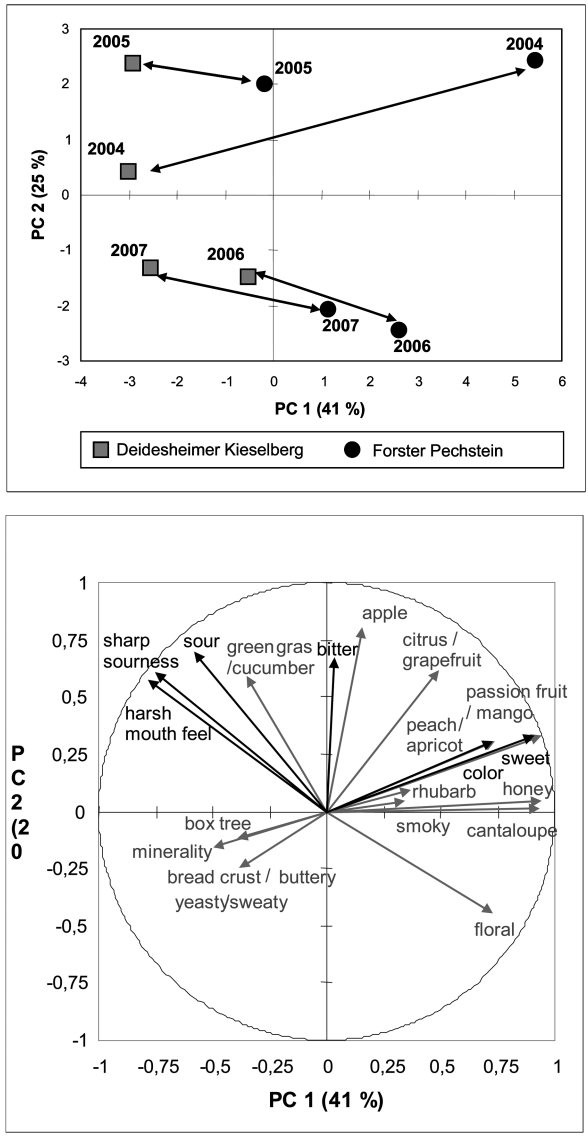

Figure 3. Sensory variation among four consecutive vintages in the vineyard sites Deidesheimer Kieselberg (sandstone) and Forster Pechstein (basalt). Upper graph: PCA scores of vineyard sites. Lower graph: PCA loadings of sensory taste (black) and odor (grey) attributes.

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If the sensory expression of terroir seems to be stable between vintages, it is still questionable if these differences will sustain the aging process. In order to compare differences between the Kieselberg and Pechstein terroirs at a young and a mature wine age, the sensory intensity ratings of the Kieselberg wines after one and four years of aging were set equal to 100% in Figure 4, while the intensity ratings of the Pechstein wines were expressed relative to the respective Kieselberg wines after one or four years of bottle age. Among 18 sensory attributes only a few really changed their relative intensity: After aging, the attributes lemon/grapefruit and smoky did not contribute to the difference between both terroirs, while the importance of the buttery odor increased. 4-Vinyl-guajacol, which is used to define the smoky standard, is not stable at wine pH and thus will degrade over four years of bottle age, explaining the sensory diminuition. While the relative sourness of the Pechstein wine increased through bottle age, sweetness and bitterness contributed less to the discrimination of both terroir wines after aging. Overall, most sensory differences however remained remarkable stable, indicating neither an increase nor an decrease of sensory distinction between both terroirs due to bottle age.

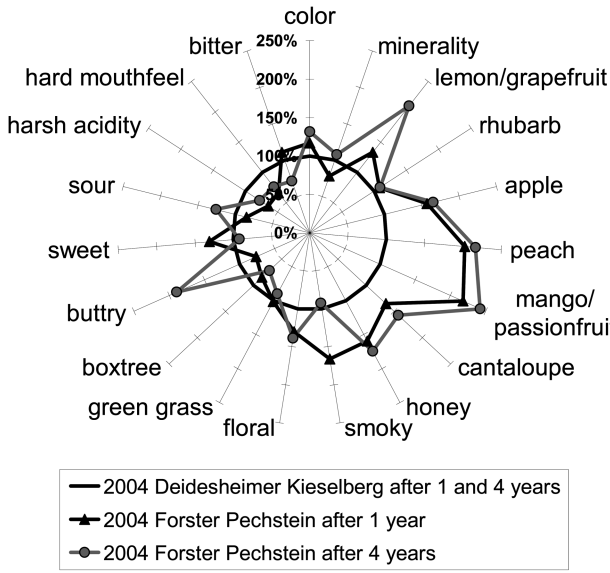


Figure 4. Comparison of sensory profiles of the wines from the bedrock types sandstone (Kieselberg) and basalt (Pechstein) after one year (n = 20 jdgs. x 3 rep) and after four years of aging (n = 13 jdgs. x 3 rep.). For both maturity levels, intensity ratings of the wines from the Deisdesheimer Kieselberg were set equal to 100%. The intensity ratings of the wines from the Forster Pechstein were set relative to this 100%-reference.

Discrimination of Types of Bedrock by Sensory Means

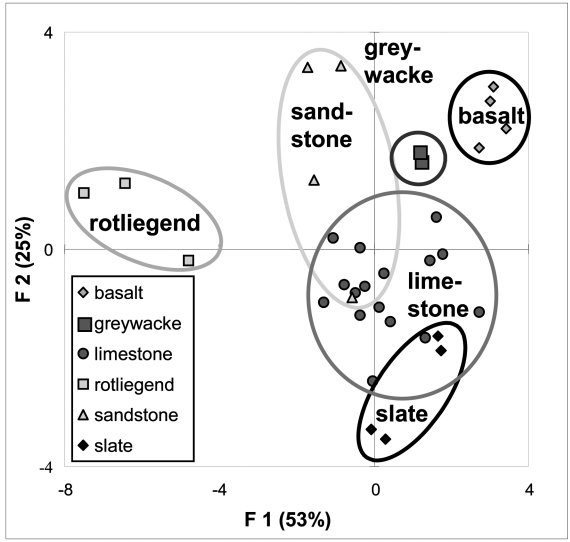
As previously mentioned, numerous publications reported on successful discriminations between wines from different geographic areas by chemical parameters as well as by sensory properties. In contrast, the experimental design of this research allowed, furthermore, to discriminate between different types of bedrock, in different Riesling growing areas within Germany. Most types of bedrock examined in this research were represented by test sites in at least two growing areas.

In a discriminant analysis of the sensory intensity ratings of all available wines produced under standardized conditions from the 2004 (N=12) and 2005 (N=21) vintages, 78% of total variability was explained by the first two factors F1 and F2 (Figure 5). The groupings of the wines originating from the bedrock types rotliegend, greywacke and basalt did not overlap with the other bedrock-clusters. There was some sensory variation between the wines from sandstone, slate, and limestone respectively, which explains the overlapping of these bedrock clusters in this discriminate analysis. However, it must be remembered that variation is not only due to different vineyard sites, but also to the climatic differences of two consecutive vintages. It is therefore astonishing, how closely, for example, the numerous wines from limestone are grouped, although they represent three different growing areas and two vintages.

For this dataset, color, sweet, bitter, hard mouth feel and the odor attributes box tree and lemon/grapefruit had the highest discriminating power, as expressed by the longest vectors in Figure 5. Wines from the bedrock type rotliegend were discriminated by their more intense yellow color, while wines from slate were perceived as being sweeter, and those coming from limestone or basalt seemed to be higher in lemon/grapefruit intensity.

The discriminant analysis was repeated with 24 wines, which were produced from the identical grape material as used for the standardized vinification. However, winemaking took place at the cooperating wine estates, adopting their commonly used winemaking practice, except for using the same yeast strain. As displayed in Figure 6 an even closer grouping was achieved, and the overall explained variance reached 93% for the first two factors. It may be speculated that winemakers being familiar with the terroir expression of their vineyards, were more successful in elaborating the typical sensory profile of different types of bedrock.

In this data set, which comprised wines from two vintages and four growing areas, slate and sandstone wines were more similar and differed from the other wines by higher minerality, and box tree and grass/cucumber odor intensities. Wines originating from limestone expressed high intensities of mango/passion fruit and peach/apricot. The wines from the bedrock type rotliegend were distinguished by higher sweetness and cantaloupe odor, while the wines originating from basalt were more bitter and displayed strong apple and lemon/grapefruit notes.



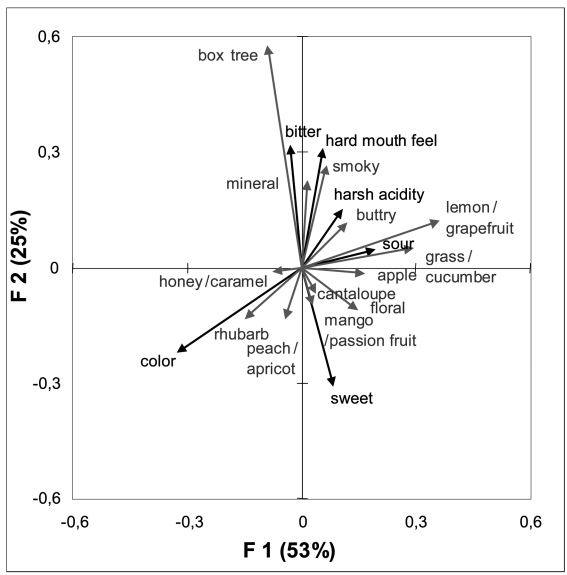


Figure 5. Discriminant analysis of different types of bedrock based on sensory intensity ratings of 2004 and 2005 Riesling wines (standardized vinification, N=33).

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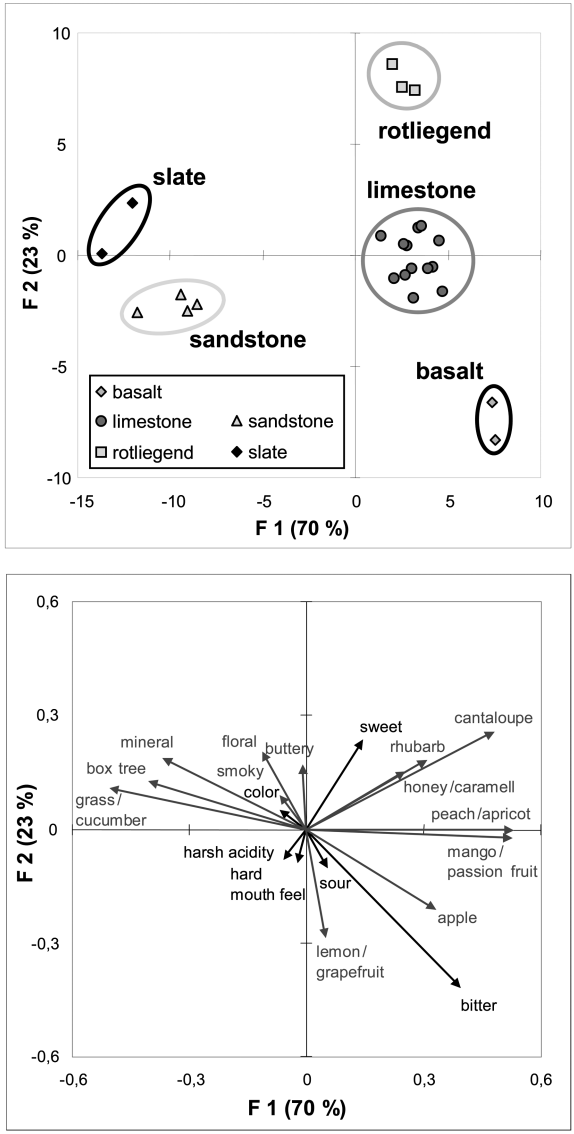

Figure 6. Discriminant analysis of different types of bedrock based on sensory intensity ratings of 2004 and 2005 Riesling wines (wine estate vinification, N=24).

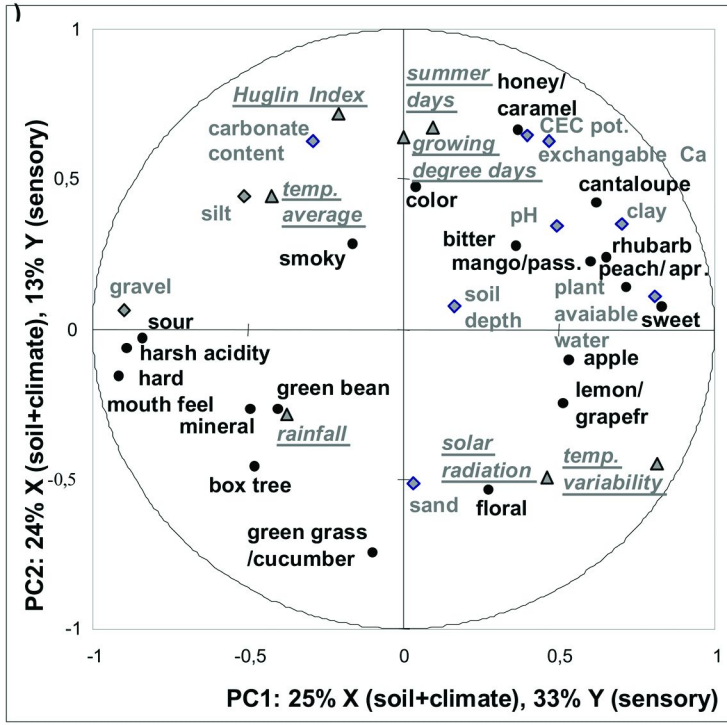

Figure 7. PLS-Regression of pedological (grey) and meteorological (grey, underlined) parameters, and sensory intensity ratings (black) of 14 2004 Riesling wines (n = 20 jdgs. x 2 or 3 rep.).

Modeling Sensory Properties with Pedological and Meteorological Data

Extensive soil analyses were conducted for the experimental sites of the Pfalz region and were combined with meteorological data from weather stations being installed nearby or right on site. These datasets were correlated with the intensities of the sensory attributes for the 2004 vintage through a Partial Least Squares-Regression (PLS). The PLS yielded a three-dimensional model, which accounted for 75.6% and 52% of explained variance in the pedo-meteorological (X) and sensory (Y) datasets, respectively. The two-dimensional PLS in Figure 7 explains 46% of sensory variance and 49% of pedo-meteorological variability.

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The upper and lower left quadrants of the sensory space are dominated by sour and vegetative attributes, while the upper and lower right quadrants display the fruity-sweet, floral and fruity-fresh (lemon/grapefruit, apple) attributes, as well as bitterness and color. The sour and vegetative attributes were associated to the contents of sand and gravel, and also to the precipitation during the ripening period following véraison. In turn, gravel content and rainfall during September and October were able to explain to a high degree sourness (R^2=0.88), harsh acidity (R^2=0.89) and hard mouth feel (R^2=0.94). The fruity-sweet attributes were correlated with the contents of clay and exchangeable Calcium, soil-pH, plant available water (PAW), potential cation exchange capacity (CEC_{pot}), Growing Degree Days (GDD), number of summer days (daily maximum temperature > 25°C), and Huglin-Index. For example, the attribute cantaloupe was explained to 76% by the parameters of GDD, number of summer days, Ca and clay content of the soils as well as PAW. For the floral and fruity-fresh attributes, the model showed associations to PAW, sand content, solar radiation, and the cumulated day-night temperature variability during the ripening period. The floral attribute yielded a R^2=0.46 based on sand content of the soil, solar radiation and night-day temperature variability during the ripening period. Due to a limited water-holding capacity of sandy soils, a mild to moderate lack of water to the grape vines can be expected in the absence of irrigation or sufficient precipitation. The formation of bound and free monoterpenes in berries causing floral aroma notes, is increased by moderately insufficient water supply (*8*). Furthermore, berries being exposed to sunlight, have been shown to have higher levels of bound and free monoterpenes than those grown in shady conditions (*28–31*).

Discrimination between Types of Bedrock Based on the Wines' Aromachemical Composition

A discriminant analysis was performed on 2004 and 2005 wines from the standardized and wine estate vinification, using a subset of 49 aroma compounds which significantly varied between the types of bedrock in an ANOVA. As displayed in Figure 8, the concentration of aroma compounds present in the head space was able to discriminate well between wines grown on the bedrock types greywacke, basalt and slate. The wines from rotliegend were well separated from those from sandstone, however they showed minor overlapping with the limestone cluster. Only the limestone and sandstone clusters overlapped to a greater extent. A further discussion of the contribution of single aroma compounds or chemical groups of compounds will follow elsewhere and will be based on a state of the art quantification via stable-isotope-dilution-analysis.

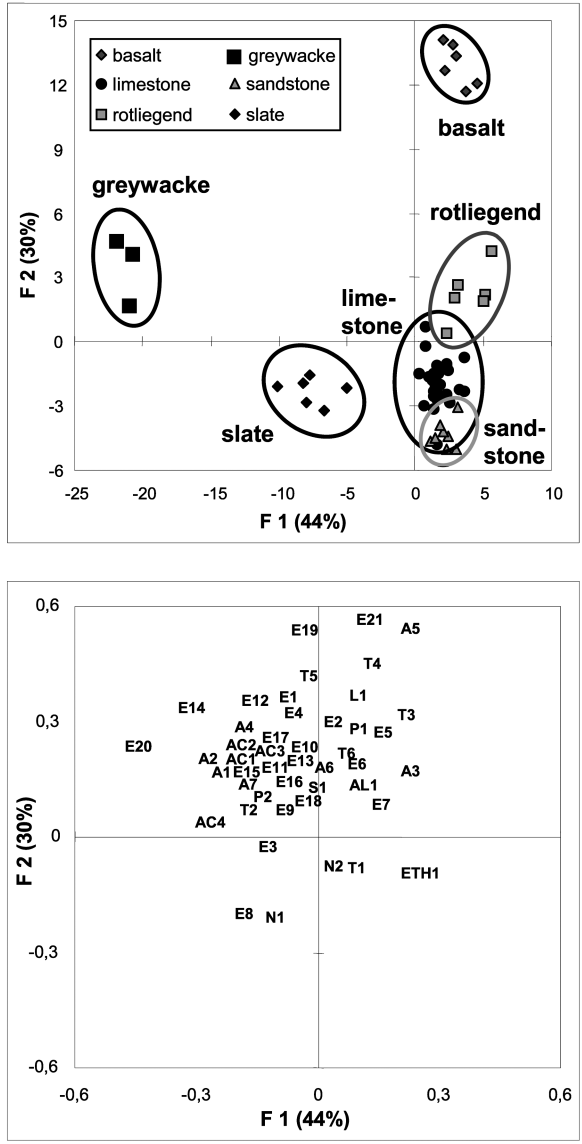

Figure 8. Discriminant analysis of different types of bedrock based on composition of aroma compounds of 2004 and 2005 Riesling wines (standardized and wine estate vinification, N=57).

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Conclusions

ANOVA and discriminant analysis showed a clear impact of terroir on the sensory properties of German Riesling, despite vintage and winemaking influences. Sensory patterns could be seen, and they varied depending on the geological substrate the wines originated from. The same could be demonstrated for aroma compounds. In a PLS, 52% of the sensory variance could be modeled with pedological and meteorological data, showing that not only geographic regions can be distinguished by sensory and chemical means, as reported in numerous publications, but also the types of bedrock, despite being distributed among several growing regions. In conclusion, terroir is indeed an important factor regarding the sensory properties of wine, and it is a true source of authenticity and typicality of wine.

During the opening speech of this symposium, a wide array of analytical methods were presented, which have allowed, over the last decade, substantial progress in authentication of foods and beverages. Although it is the main goal of these efforts to detect adulteration, protect consumers against fraud and verify products with protected labels, it is interesting to note that one analytical method was not mentioned at all, which comprises the predominant tool consumers apply to assess integrity and quality of food: sensory analysis. It may be encountered that "sensory analysis relies on attributes which are subjective, very difficult to measure and usually not regulated by law" (S. Sforza, personal communication during the symposium). However, this criticism is also valid for all non targeted analytical approaches, where fuzzy pattern recognition replaces the precise analysis of a single compound. Therefore, sensory analysis should be viewed as an important non targeted approach, where our peripheral senses and central brain processes apply structure-recognition techniques to identify authenticity and typicality of food products, beverages and in wine even the impact of various terroirs.

Acknowledgments

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Chapter 10

Analytical and Multivariate Statistical Methods for Differentiation of Wines Produced with Oak Chips and Barriques

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Due to the increasing consumer demand for oaked wines, wine makers have increased the production of such wines. The traditional method of aging wines in oak barrels (barrique), however is very expensive and it is more profitable to use an oak chips treatment. In the European Union the use of oak chips is restricted, e.g., in Germany for high quality wines. In general, wines labelled with barrel or barrique aging may not be treated with oak chips. For consumer protection and competitive distortions, it is necessary to have sophisticated analytical methods for authentication of barrel aged wines and to prove an oak chips treatment repectively. A method was developed using solid phase extraction (SPE) and gas chromatography-mass spectrometry for quantification of 20 volatile compounds in 138 authentic wines produced with both oenological practices. In a second approach SPE extracts of selected wines were analyzed with ^{1}H-NMR to check for differences in the ^{1}H-profiles of barrel aging and oak chips treatment. The data of both analytical methods were evaluated with multivariate methods

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using PCA and LDA. The first statistical models showed that a differentiation of both oenological practices was successful.

Introduction

The use of oak wood (quercus species) in wine production to influence the sensory properties of wine towards a typical oak flavour and taste can be achieved by different technologies. Fermentation of grape and/or several months storage of the wine in oak casks, especially small 225 liter toasted oak casks (barrique) is the traditional procedure, whereas a treatment with toasted oak pieces (oak chips) or other wooden arrangements like staves is the faster and less expensive procedure.

The use of oak chips as an oenolocial practice was first allowed in the European Union in 2006. The actual regulations EC 606/2009 appendix 9 (*1*) and EC 607/2009 appendix XVI (*2*) lay down different rules and specifications for the use of oak wood in wine production and the declaration of an oak treatment. Thus any declaration or indications of a production with or in an oak or a Barrique barrel is not allowed, if oak chips or other oak materials are used. To control this regulations it is necessary to develop sophisticated analytical methods.

There are many studies on the composition and ingredients of toasted oak wood, differences in geographical origin and oenological technologies using oak in wine production (*3*–*8*). However, only a few systematical studies on analytical possibilities for differentiation of oak cask and oak chips exist. The main volatile components which may migrate from toasted oak into the wine are phenolic compounds (e.g., 2,6-dimethoxyphenol), furanic derivatives (e.g., furfural), aromatic aldehydes (e.g., vanillin) and lactones (e.g., E/Z-whiskeylactone) (*9*, *10*).

The difficulty of a significant discrimination of wines stored in oak barrels and its differentiation from wines treated with oak chips is caused by the fact that there are no significant qualitative differences or impact compounds within the rather complex composition of oak wood. Thus a significant differentiation is an analytical challenge which can only be achieved by a multivariate statistical exploration of quantitative differences within the pattern of specific oak compounds in wine. These differences can be caused by specific diversities of the production and toasting methods of chips and barrique; oak and barrique casks are toasted by a direct contact of fire, whereas chips are toasted flameless at constant temperatures. Further parameters which probably influence the pattern of compounds from oak in wine are the contact time of the wine with the oak. According to the European legislation red barrique wines have to be stored in the barrel for at least six months and white wines at least for three months. In contrast, oak chips are in contact with the wine for about two to eight weeks. The ratio of oak surface to wine volume as well as physical or chemical changes due to a micro-oxygenation are further aspects which can be responsible for an analytical differentiation.

On the basis of selected volatile compounds Arapitsas et al. (*11*) as well as Butticaz et al. (*12*) showed that a differentiation between chips and barrique treatment is possible.

In our studies the discrimination was focussed first on the analysis of specific volatile compounds of native and toasted oak wood using gas chromatography-mass spectrometry (GC-MS) in authentic wines produced with different commercial oak chips and barrique casks.

In a second approach ^{1}H-NMR spectra from extracts of the same wines were analyzed and evaluated using multivariate statistical analysis based on pattern recognition. The aim of this method was to find non-targeted differences in the pattern of these wines. It is assumed that by non targeted ^{1}H-NMR mainly non volatile compounds are detected and used for analytical differentiation (*13–16*).

The main goal of this preliminary study, integrated in a long-term project, was to develop initial statistical models for both analytical methods to differentiate wines produced with oak chips and wines aged in barrique. This is why we started with a limited amount of authentic wines and commercial samples.

Material and Methods

Wines

Red and white wines were produced from grapes of vintage 2006 and 2007 in the German wine growing region Franconia at technical scale at the Bayerische Landesanstalt für Weinbau und Gartenbau, Veitshöchheim. A group of these wines was aged in oak barrels (five red wines and eleven white wines) while another group was treated with oak chips (61 red wines and 59 white wines). These wines were used as authentic wines.

Ten white wines were produced with barrel aging, oak chips and simultaneous use of both practices in another winery for the purpose of a thesis (*17*). These wines where used as authentic wines to check the statistical model.

Thirty-three samples labelled as barrique wines were taken from the official Franconian wine inspection authority and fourteen commercial wines treated with oak chips were taken from the local market. These commercial wines were used to check the statistical models developed on the basis of the authentic wines.

Oak Materials and Treatment

For production of authentic wines produced with oak chips, samples from different geographical origin (USA, France, Germany), with different toasting degrees (light, medium, heavy), and with different sizes (small, medium, large) were obtained from German specialist retailer for oenological equipment. According to common oenological practice a concentration of 2.5 g/L and a contact time in stainless steel barrels of six weeks was used. For production of barrique wines new medium and high toasted small oak barrels (volume 225 L), produced from American, French and German oak wood, were used; the wines were stored in the barrels for six months. After oak treatment all wines were bottled in the wine cellars.

Analytical Methods

Gas Chromatograph-Mass Spectrometry (GC-MS) Analysis of Oak Wood Volatile Compounds

Volatile oak compounds were separated from the wines with LiChrolut EN (Merck, Darmstadt, Germany) solid phase extraction (SPE) cartridges. Di-chloromethane (rotisolv pestilyse, Roth, Karlsruhe, Germany), methanol (HPLC LGC, Middlesex, U.K.), and a solution of 13 % vol ethanol (99.7 – 100% v/v, VWR international Ltd.), West Chester, PA, USA) were successively used for equilibration of the adsorbent. The wine sample (50 mL), spiked with the internal standard compounds 2-octanol (ABCR, Karlsruhe, Germany), ε-decalactone (Alfa Aesar, Heysham, U.K.) and methyl cinnamate (Sigma-Aldrich, St. Louis, MO, USA), was eluted through the cartridge by means of a vacuum pump. The cartridge was dried by air for 15 minutes. Volatile compounds were eluted with 1 mL dichloromethane, according to the method of López et al. (*18*).

GC-MS was performed with a Varian 3900 gas chromatograph coupled with a Varian 2100T Ion Trap MS. One µL of the Lichrolut eluate was injected at 240 °C with a split ratio of 1:10 on a Agilent DB-5MS column (5 %-phenyl-95 %-dimethyl-polysiloxane; 30 m × 0.25 mm i.d.; df 0.25 µm). Carrier gas was helium with a constant flow of 1 mL/min. A GC-oven temperature program, was used starting at 50 °C; temperature was raised at 3 °C/min to 80 °C, 10 min at 80 °C, then at 5 °C/min to 240 °C, 5 min at 240 °C.

The mass spectrometer was operated in full scan mode (EI-ionization, mass range m/z 40-400) with a solvent delay of 2.0 min.

The identification of the twenty volatile compounds was performed via retention time and selected mass-to-charge ratios (m/z) of the single compounds. Quantification was performed by an internal standard method after integration of the areas of selected m/z signals. Peak identification and integration were checked with corresponding full scan signals.

Calibration was performed using reference substances with GC-purity obtained from different suppliers (Roth, Karlsruhe, Germany; Sigma-Aldrich, Steinheim, Germany; Riedel de Haen, Seelze, Germany; Merck, Darmstadt, Germany; ABCR, Karlsruhe, Germany). The reference substances were spiked prior to SPE in a non-oaked standard white wine as a blank sample. The subtraction of the blank concentrations was done before calibration. A multilevel calibration (7 to 9 levels) was used for each compound, according to its expected concentration range in oaked wines.

Non-Targeted ^{1}H Nuclear Magnetic Resonance Spectroscopy (^{1}H-NMR)

A selected number of 27 authentic reference wines and 10 test wine samples were extracted by HR-P solid phase extraction (SPE) cartridges (Macherey-Nagel, Germany). For equilibration the SPE cartridge was rinsed with 3 mL methanol (99 %, HPLC LGC, Middlesex, U.K.), followed by 3 mL of 13% vol ethanol (99.7-100 % v/v, VWR International Ltd., West Chester, PA, USA). A mixture

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of 20 mL of wine sample with 2.2 mL of KH_2PO_4–buffer (min. 99.5 %, RdH Laborchemikalien GmbH + Co. KG, Seelze, Germany) (adjusted with H_3PO_4 to pH 2.0) was spiked with 0.1 % TSP (trimethylsilyl-2,2,3,3-tetradeuteropropionic acid, Merck, Darmstadt, Germany) and eluted through the SPE-cartridge by means of a vacuum pump. The cartridge was dried by air using a slight vacuum for 15 minutes and eluted with 1.5 mL methanol-d_4 99.8 atom % D (Roth, Karlsruhe Germany).

^{1}H-NMR spectra were recorded with a Bruker 400 MHz Avance AVIII NMR Spectrometer equipped with TOPSPIN 2.1 Patch Level 5 software, a Bruker ^{1}H/^{13}C inverse gradient shim probehead with ATMA, Z-gradient and BTO 2000 temperature unit and a BACS 60 sample changer. The Bruker shimsystem BOSS II and a BCU 05 temperature unit for temperature regulation were used.

Temperature calibration (300.0°K) of the probehead was performed with methanol-d_4 (99.5 %) according to the method of Findeisen et al. (*19*). Sample measurement started after 5 min temperature equilibration (5 min), ATMA, Lock and shimming procedures (Topshim routine). Using an automatic sequence first the O1 frequency for water suppression was determined with a 1D zgpr-experiment followed by a 1D NOESY experiment (NS = 32, DS = 4, SW = -5.4327 – 15.0860 ppm, Lb = 3)

The ^{1}H spectra were processed with Bruker APK0.NOE processing routine (Fourier transform, phasing 0.order). Since a digital filter "Baseopt" was used, no baseline correction was necessary. The resulting ^{1}H-spectra were used for multivariate analysis without further pre-processing.

Statistical Methods

Multivariate Anaylsis of Volatile Compounds

The multivariate analysis of the concentrations of volatile compounds was performed with SPSS® Statistics 16 and WinSTAT for Microsoft® Excel 2007. The software packages were used for development and calculation of statistical models on the basis of principal component analysis (PCA) and linear discriminant analysis (LDA). For the statistical analysis the samples were divided into the groups: barrique wines, oak chip wines and wines with simultaneous use of barrique and oak chips.

All data was standardized and mean centered before application of multivariate statistical analysis. AptPlot 6.3.4 was used to plot the graphs.

Multivariate Analyis of ^{1}HNMR Spectra

Bruker AMIX 3.6 software package was used for multivariate analysis of the ^{1}H spectra.

The spectra were assigned to two groups, the oak chip group and the barrique group. Each spectrum was transformed into fragments, using the method simple rectangular bucketing between 5 and 10 ppm with 0.04 ppm for each bucket. The

fragments, so-called buckets, were summarized in a bucket table where each row refers to a spectrum (sample) and each column to a bucket (*21*).

Results and Discussion

GC-MS

The results in Table I show that it was not possible to find significant differences between oak chip wines and barrique wines by means of the concentrations of the twenty volatile oak wood compounds found in the authentic wine samples, since the standard deviations of nearly all volatile compounds were higher than the mean values. In addition, there was no impact compound which could be attributed to chips or barrique only.

A more representative sample of the population could be achieved by taking more samples (wine oak materials) and different oenological treatments into account. However, the high standard deviation of the data in Table I is also a result of the large variability of toasted oak wood barrels and chips with regards to their different geographic origins and manufacturing techniques, especially the different and non-standardized toasting practices.

According to the results in Table I a monovariate differentiation based on single values would not lead to a significant discrimination of both groups.

Thus only a multivariate statistical approach seemed to enable a discrimination of chip and barrique wines. In this case a PCA analysis of the GC-MS results of the authentic wines was done first to get an overview of the variance of the samples. The results of the PCA also gave information about the outliers and the structure of the data matrix. Next a LDA was done with the same samples. Before analysis the wine samples were divided into the two groups, chip and barrique wines. Since a two group LDA results in only one discriminant variable the plot was created using the results of the PCA.

For visualizing the results a two dimensional plot was created using the results of PCA and LDA (Figure 1). In this plot the discriminant variable from the LDA is displayed versus the first principal component from the PCA. As is demonstrated in Figure 1, the two groups (oak chip wines and barrique wines) could be clearly separated.

A leave-one-out cross-validation was used to check the significance of the statistical model. The results in Table II show that all oak chip wines were predicted into the correct group. Only two (12.5 %) of the barrique wines were misclassified. Although we have been aware of the numeric disparity of both groups and the fact that more barrique samples should have been be taken into consideration, this initial discrimination model already showed that a validated differentiation of barrique and oak chip wines is feasible and that it is worth to extend this model with more authentic and commercial samples.

Table I. Mean values and standard deviation of selected volatile compounds in authentic wines produced with oak chips and with barrique storage

Compound	*Wines produced with oak chips (n =120)*		*Wines produced with barriques (n = 16)*	
	mean [µg/L]	*standard deviation*	*mean [µg/L]*	*standard deviation*
furfural	683	668	2876	3499
furfurylalcohol	449	615	2535	4964
5-methylfurfural	39	57	277	326
4-ethylphenol	41	170	47	179
guaiacol	4	1	7	13
4-methylguaiacol	2	4	7	27
5-hydroxymethylfurfural	79	169	128	345
4-ethylguaiacol	162	27	634	789
E-whiskeylactone	32	26	33	29
5-acetoxymethyl-2-furaldehyde	2	3	4	4
Z-whiskeylactone	18	23	19	35
2,6-dimethoxyphenol	68	215	53	24
eugenol	28	21	87	113
vanillin	164	232	9	165
isoeugenol	99	123	112	124
acetovanillone	32	29	18	18
δ-decalactone	21	25	30	25
ethyl vanillate	189	195	155	163
2,6-dimethoxy-4-(2-propenyl)-phenol	3	16	12	8
syringaldehyde	98	116	467	494

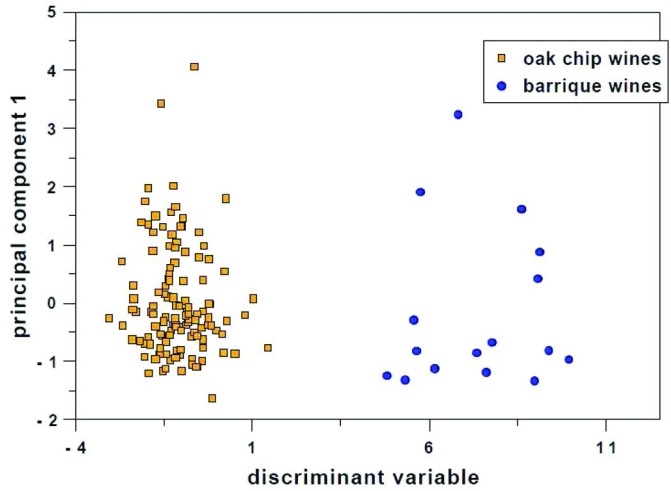

Figure 1. LDA-PCA plot: Discrimination of authentic wine samples produced with barrique and oak chips by means of LDA using 20 volatile compounds in the model.

Table II. Cross validation of the linear discriminant analysis of authentic wine samples

cross validation		*predicted group*	
	group	*oak chips*	*Barrique*
number	oak chips	120	0
	barrique	2	14
%	oak chips	100	0
	barrique	12.5	87.5

The model shown in Figure 1 was additionally validated with ten test wines (consisting of two different white wines with five different oenological treaments each). In Figure 2 it is obvious that the six test oak chips wines and the two test barrique wines were classified to the correct group of the model. The two wines which had been stored in a barrique cask (2nd layer) by simultaneous oak chip treatment were predicted to the barrique group. The position of the two samples in Figure 2 indicates that they are at the borderline of the barrique group. One reason for this result could be the influence of oxygen during storage in a used barrique (*20*).

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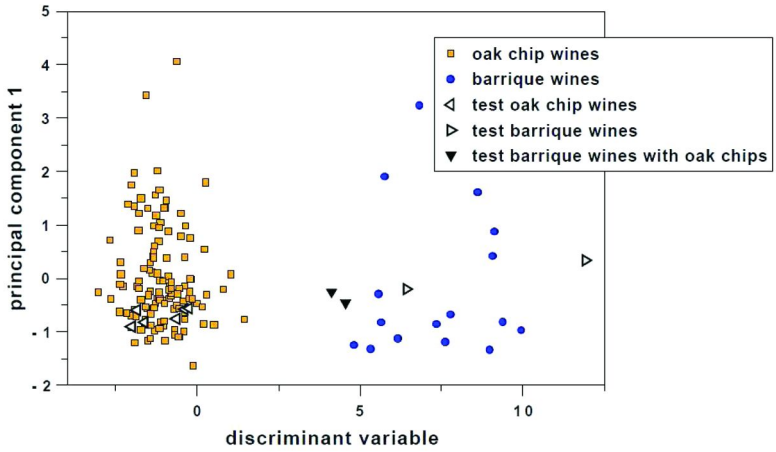

Figure 2. LDA-PCA plot: Validation of the discrimination model with an independent test set of ten different oaked wines (test wines).

Finally, thirty-nine commercial German wine samples were analyzed. It was known from the application forms of the official wine testing that thirty-one samples should have been stored in Barrique and eight samples were treated with oak chips. The evaluation with our GC-MS data model showed a correct classification for both types of wine (Figure 3). The results also showed that the two barrique wines treated simultaneously with oak chips (Figure 2) were in between the commercial barrique wine group. Thus a differentiation of wines which are produced with a simultaneous use of oak chips and barrique is not possible via the analysis of volatile compounds from oak with this model.

The results show also that the barrique wine group scatters more than the oak chip group. One reason could be the fact that oak chips are toasted with more moderate and standardized procedures than oak barrels which are toasted over an open flame.

The results plotted in Figures 1 to 3 confirmed that a classification of oak chips and barrique is feasable by means of linear discriminant analysis of selected volatile compounds in oaked wines. To improve the significance of the model, the data base with authentic wines has to be extended e.g., with barrique wines produced in different countries, oak from other geographical origins, wines which have been fermented in barrique and with oak chips respectively, or wines produced with oak staves.

In order to classify wines produced in barriques with simultaneous use of oak chips correctly, we had to develop further analytical tools.

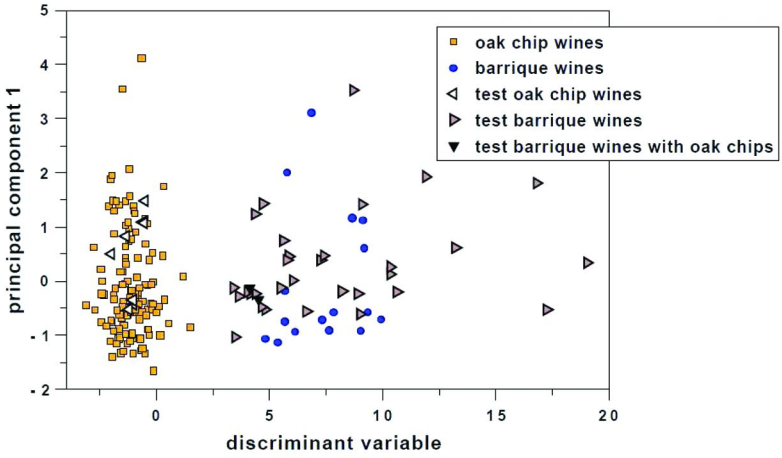

Figure 3. LDA-PCA plot: Classification of 39 commercial oaked wines using the discrimination model.

^{1}H-NMR

Water-suppressed ^{1}H-NMR spectroscopy in combination with chemometrics is a powerful tool with regard to differentiation and classification of geographical origin, aging or other issues of authentication. Thus this analytical technique was used to check for the first time a discrimination of the wines stored in barrique and wines produced with oak chips. For this analysis all spectra had to be comparable in terms of chemical shift which was provided by adjusting the pH of each sample by adding the buffer solution before SPE and by adjusting the temperature to an exact level (300.0 K ± 0.1).

SPE was performed to enrich the minor non-volatile compounds typical for oak and to separate ethanol and the major part of water from wine before ^{1}H-NMR measurement.

The ^{1}H-NMR spectra of twenty-seven authentic wine samples selected from both groups (eighteen barrique wines and nine oak chip wines) were analyzed using pattern recognition techniques.

The spectra were assigned to two groups, the oak chips and the barrique group. After transformation of each spectrum into buckets (*21*) a principal component analysis was used to identify where the buckets with the highest information are localized and to detect differences between the groups.

The most important ^{1}H signals were in the aromatic region with a chemical shift between 5 ppm and 10 ppm. This seems to be reasonable, since in this region of the spectrum signals of phenolic and polyphenolic compounds from wood should be localised (*22*).

The results of the principal component analysis of the buckets is presented in Figure 4; the wines with barrique storage and oak chips treatment could be separated very well by ^{1}H-NMR profiling. The highest information for this

separation was assigned by principal component 1, whereas the information on the other principal components was negligible.

To control this statistical model, four wines produced with oak chips, four wines produced with barrique and two wines produced simultaneously with barrique and chips were analyzed by ^{1}H-NMR. In Figure 5 it is shown that the oak chip wines and the barrique wines were classified correctly. The two test wines which had been oaked simultaneously with barrique and oak chips were clearly separated from the barrique and the chips group. This could not be achieved with the discrimination model on the basis of volatile compounds. The discrimination of the variant 'barrique with chips' with ^{1}H-NMR must be verified with further samples.

In summary, the results presented in Figure 5 and 6 encourage us to continue with ^{1}H-NMR methods for classification of wines treated with different oaking practices. In order to reduce the complex sample preparation with SPE a direct measurement of wines using multiple solvent suppression of water and ethanol will be used in future. Further studies are necessary to identify the compounds which are responsible for the discriminating ^{1}H-signals.

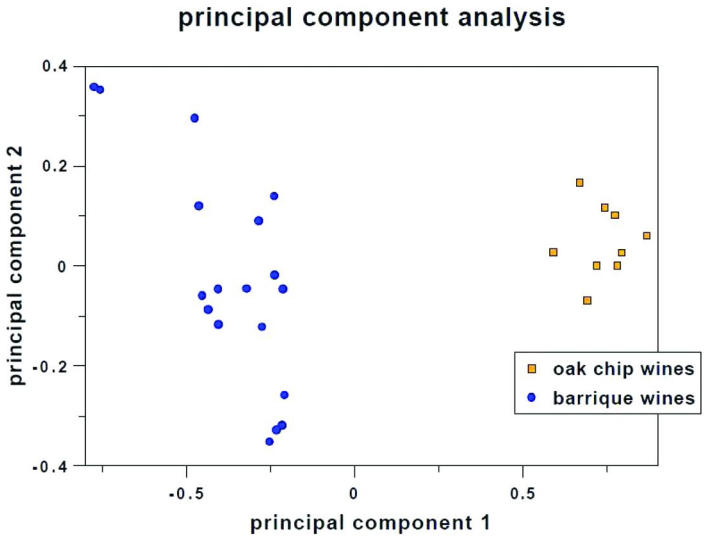

Figure 4. Discrimination of selected authentic wine samples produced with barrique and oak chips by means of PCA using signals of ^{1}H-NMR spectra between 5 and 10 ppm.

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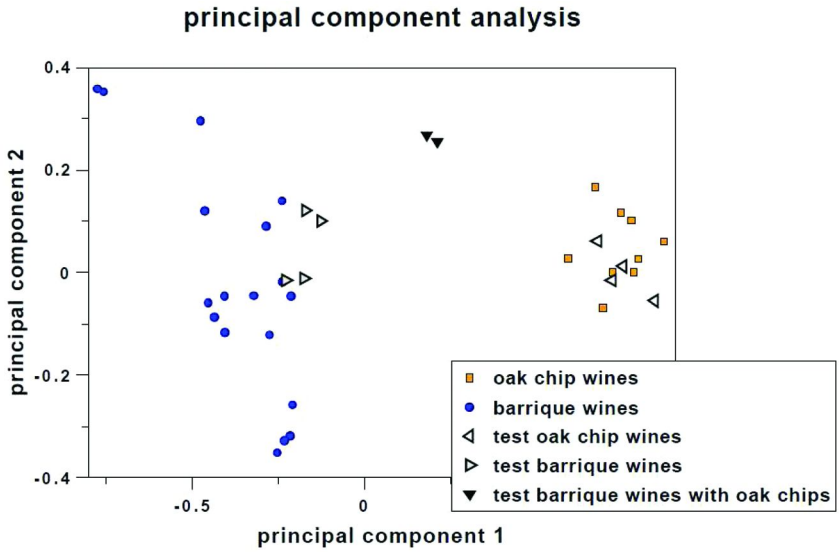

Figure 5. Validation of the PCA-discrimination model using signals of [1]H-NMR spectra between 5 and 10 ppm with an independent test set of different oaked wines (test wines).

Conclusion

For authentication and classification of oaked wines two preliminary multivariate discrimination models were developed on the basis of concentrations of volatile compounds determined with GC-MS as well as on the basis of selected signals of [1]H-NMR spectra. The discrimination models were based on authentic wine samples and validated via cross-validation and independent test wines. A classification of barrel (barrique) aging and oak chip treatment was possible with both models, whereas a detection of two wines produced with a simultaneous treatment of oak chips and barrique was only feasible with the [1]H-NMR.

Further authentic and commercial wines, taking into consideration also further oenological oak practices, will be analysed with both analytical methods to extend the data pool and to achieve a more significant and representative discrimination and classification of oaked wines.

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Chapter 11

Correlation between the Concentrations of Two Oak Derived Key Odorants and the Intensity of a Woody-"Barrique-Type" Odor Note in Different Red Wines

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During barrel aging, in particular, odorants like cis- and trans-whiskylactone, vanillin and several phenols are released into the wine. However, although in aroma profile analyses odor qualities like woody, vanilla or coconut-like are often used to describe the "*barrique*-type" aroma, a clear correlation between the intensity of such aroma qualities in the overall profile and the concentrations of key odorants, often displaying similar odor qualities, is scarcely available. On the basis of a comparison of the key odorants in a Dornfelder red wine aged in barrels or steel tanks, respectively, this study attempted to correlate, in particular, the intensity of the "*barrique*" aroma in some commercial red wines with the concentrations of cis-whiskylactone and vanillin.

The aroma is an important quality attribute of wine, and thus, the identification of volatile compounds in either white or red wine has been a research topic of numerous investigations in the past. So far ~400 volatiles have been identified (*1*). It is well-accepted in the literature that, besides the grape variety, the process of wine manufacturing and storage has a clear influence on the overall aroma of the final product, and in particular, red wines stored in oak barrels (*barrique*-type) are often rated by the consumer to be of higher quality. The term *"barrique"* is French

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and means small wine barrel, which, by definition, is a wooden barrel with a small volume, typically 225 liters (*2*). The most frequently applied wood in cooperage is oak imparting the typical sweet-woody or *barrique*-type aroma to wines stored in oak barrels (*3*, *4*). Several compounds have been suggested to contribute to the typical aroma of barrel aged wine, and cis-whiskylactone and vanillin are among the most addressed volatiles (*5*, *6*).

Concepts of molecular sensory science, such as GC-olfactometry, the calculation of odor activity values and, finally, aroma recombination experiments have been proven as useful tools to characterize the key aroma compounds of foods (*7*). The application of such concepts on, e.g., white wine (*8*), Bourbon whisky (*9*) or tea (*10*) have recently shown that only a limited number of several key odorants are able to interact with the odorant receptors in the human olfactory organ during food perception, thus creating the final aroma in the brain. On the basis of such results, it has, however, turned out that, although often only about 20-30 key odorants are able to mimick the typical aroma of the respective food, usually none of them elicits an odor close to the odor of the food itself.

As previously shown by sensory experiments, the human olfactory system is not able to detect the presence of a single constituent on the basis of its odor quality in mixtures containing more than three odorants (*11*). The fact that single odorants cannot be identified in a mixture seems to be a limitation of human aroma perception, however, obviously the sense of smell provides a means to identify quite complex mixtures of aroma compounds within seconds on the basis of a certain aroma profile. Thus, because sensory panels are able to describe the intensities of single aroma qualities when evaluating food aromas, it is a challenge in flavor science to correlate the odor qualities detected by a sensory panel with the presence or the concentrations, respectively, of single key odorants.

Using the concept of molecular sensory science (*7*), in a recent study, the key odorants present in a Dornfelder red wine manufactured as *barrique*-style were identified and compared to those present in a red wine from the same batch, but stored in a steel tank (*12*). In particular cis-whiskylactone and vanillin, but also four aroma-active phenols showed higher concentrations in the barrel aged wine (*12*). However, although these aroma compounds have been identified as volatile constituents in red wines several times before (*5*, *6*), their contribution to the overall aroma of red wines manufactured by the "*barrique*" method is still not clear. Thus, the aim of the present study was to elucidate whether a correlation between an overall "sweet-woody" odor note in barrel aged red wines and, in particular, cis-whiskylactone and vanillin does exist.

Experimental Part

Wines

Five commercial barrel aged red wines of different origin and varietal were purchased from a local wine store: A Pinot Noir from Germany (vintage 2007), a Cabernet Sauvignon from Chile (vintage 2007), a Bordeaux from France (vintage 2004), a Rioja from Spain (vintage 2003) and a Barolo from Italy (vintage 2001).

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In addition, a Dornfelder red wine was obtained from a winegrower in Saulheim (Rheinhessen), Germany. This wine (vintage 2008) had either been stored in oak barrels or had been stored in steel vessels, respectively.

Aroma Profile Analysis

Aroma profile analyses were performed by a trained sensory panel consisting of 14 panelists as previously described (*12*). The following aroma descriptors, represented by the compound given in parentheses, were chosen for sensory evaluation, and their intensities were ranked on a seven-point scale (steps of 0.5) from 0 (not perceivable) to 3 (strongly perceivable): flowery (2-phenylethanol), malty (3-methyl-1-butanol), fruity (ethyl 3-methylbutanoate), cooked apple-like ((2E)-1-(2,6,6-trimethyl-cydclohex-1-en-1-yl)but-2-en-1-one), clove-like (4-allyl-2-methoxyphenol), sweaty (3-methylbutanoic acid), smoky (2-methoxyphenol), vanilla-like (4-hydroxy-3-methoxy-benzaldehyde), coconut-like ((4S,5S)-5-butyl-4-methyldihydrofuran-2(5H)-one; cis-whiskylactone), vinegar-like (acetic acid), butter-like (2,3-butandione), and cooked potato-like (3-(methylthio)-propanal). The judgments of the panelists were averaged.

Simulation of the *Barrique*-Type Aroma Note in the Dornfelder Red Wine

A Dornfelder red wine, which had been stored in steel vessels, was spiked with five odorants: cis-whiskylactone, vanillin, 4-ethyl-2-methoxyphenol, 2-methoxyphenol and 4-ethylphenol. The amounts of the five odorants added were chosen in the way that the concentrations in the resulting wine were the same as in a wine of the same batch, which had been stored in oak barrels. The overall similarity of the aroma was ranked as described recently (*12*).

Sensory Evaluation of the *Barrique*-Type Aroma Note in Commercial Red Wines

The intensity of the *barrique*-type odor quality in five commercial red wines was ranked by a trained sensory panel consisting of 18 panelists. The panel ranked the intensity of this note in the wines using an eleven step scale from 1 to 5. As a reference, the odor descriptor for the *barrique* aroma was defined as "sweet-woody", and was represented by a model solution of cis-whiskylactone (2.2 mg/L) and vanillin (15.9 mg/L) in ethanol/water (13%).

Quantification by Stable Isotope Dilution Assays (SIDA)

Wine (0.5 mL - 400 mL), depending on the amount of the respective odorant present, was spiked with isotopically labeled vanillin and cis-whiskylactone, respectively, and after equilibration, extracted with diethyl ether. After drying over Na_2SO_4, the volatiles and the internal standards were isolated by SAFE distillation, and the analytes were quantified by mass chromatography as recently reported (*12*).

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Sensory Study on the Correlation of the Concentrations of Vanillin and cis-Whiskylactone with the Intensity of a "Sweet-Woody" Note

Solutions of either vanillin or cis-whiskylactone, respectively, dissolved in 13% ethanol in water, were presented to the sensory panel in increasing concentrations. In triangle test, either one or two samples contained the double concentrations of the reference, and the panel had to identify the vessel containing the higher concentration. The evaluation of the results was done according to § 64 LFGB method BVL L 00.90-7 appendix A.1 (*13*).

Results

In a first sensory experiment, the aroma profiles of the barrel aged Dornfelder red wine and of the same wine aged in a steel tank were determined. The data showed that, in particular, the vanilla-like and the smoky-sweet but also the coconut-like aroma notes were higher in the barrel aged wine (Table I).

Quantitation of the most odor-active compounds in both wines revealed no significant changes for most odorants, but, in particular, cis-whiskylactone, vanillin and three phenols were much higher in the barrel aged wine (*12*).

Table I. Comparison of the aroma profile analysis of the Dornfelder red wine aged in an oak barrel (OB) or a steel tank (ST)

Odor quality	*Intensity*	
	OB	*ST*
Fruity	2.0	2.0
Flowery	1.6	1.6
Malty	1.5	1.1
Cooked apple	1.8	1.1
Vanilla-like	**1.7**	**0.4**
Smoky-sweet	**2.3**	**1.0**
Clove-like	**2.1**	1.0
Coconut-like	**1.0**	0.4
Sweaty	0.7	1.3

One option to test the contribution of oak derived odorants to the overall "*barrique*-type" aroma of a wine is spiking wine from the same batch, which has been stored in steel barrels, with reference compounds matching their natural concentrations in wine of the same batch, which had been stored in oak barrels. This experiment was performed using the quantitative data for two Dornfelder red wines of the some batch either stored in steel tanks or in oak barrels, respectively (Table II).

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Table II. Concentrations of five selected odorants in Dornfelder red wine differing in their concentrations after aging in steel tanks (ST) or oak barrels (OB), respectively[a]

Odorant	*Conc. (μg/L)*	
	ST	*OB*
cis-whiskylactone	<0.1	58.6
vanillin	8.6	256
4-ethyl-2-methoxyphenol	0.2	5.8
2-methoxyphenol	3.1	6.9
4-ethylphenol	3.1	92.3

[a] Data are taken from (*8*).

By increasing the concentrations of the five compounds in the wine stored in steel tank up to those in the barrel aged wine led to an increase, in particular of the vanilla-like, smoky-sweet, clove-like and coconut-like odor notes in the red wine from the steel tank (data not shown). Overall, the aroma clearly mimiced the *barrique*-type aroma note of the wine stored in oak barrels (*12*).

Correlation Between Vanillin and cis-Whiskylactone and the Woody Odor Note in Red Wine

To evaluate the contribution of, in particular, vanillin and cis-whiskylactone to the overall woody, *barrique*-type aroma of red wine, next the following sensory experiments were performed: The panel was trained for a sweet-woody (*barrique*-type) aroma note using a mixture of cis-whiskylactone (2.2 mg/L) and vanillin (15.9 mg/L) in a 13% aqueous ethanolic solution. The overall aroma of the binary mixture was clearly described as sweet-woody. Using this reference, the panel evaluated the intensity of the "woody" odor note in five commercial red wines. As a "zero" control, the Dornfelder wine stored in steel barrels was used. The data showed the lowest intensity of the "*barrique*-type" aroma for the German Pinot Noir (Figure 1), while the Cabernet Sauvignon from Chile, the French Bordeaux and the Portuguese Rioja were ranked with the most intense woody-sweet odor.

To evaluate their role in the overall aroma, cis-whiskylactone and vanillin were quantified by stable isotope dilution assays in the five wines (Table III). The concentrations of cis-whiskylactone were lowest in the German Pinot Noir, and in the Barolo red wine. On the other hand, particularly compared to the Pinot Noir, the lactone was by factors of ~40 or ~75, respectively, higher in the Bordeaux and the Rioja wine (Table III). The Pinot Noir also showed the lowest concentration of vanillin, while the Rioja showed the highest concentration of this odorant.

It is a common agreement in odor psychophysics that aroma compounds must occur in concentrations above their odor thresholds in order to contribute to a given aroma. This fact is commonly addressed by the odor activity value (OAV: ratio of concentration to odor threshold; (*7*)). A calculation of the OAVs of the two odorants on the basis of their odor thresholds in ethanol/water revealed

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that in the Pinot Noir wine both odorants were present far below their odor thresholds (Table IV). These data are in good agreement with the low intensity of the "*barrique*-type" aroma quality in this wine (Figure 1). On the other hand, both compounds exceeded their odor threshold in the Rioja, the Bordeaux as well as the Cabernet Sauvignon wine, suggesting that both odorants might contribute to the overall woody, *barrique*-type odor detected in these wines (Figure 1). However, in the Rioja the OAVs were much higher than in the Bordeaux and the Cabernet Sauvignon wine, although no clear difference in the intensity of this odor note between the three wines was detected (Figure 1).

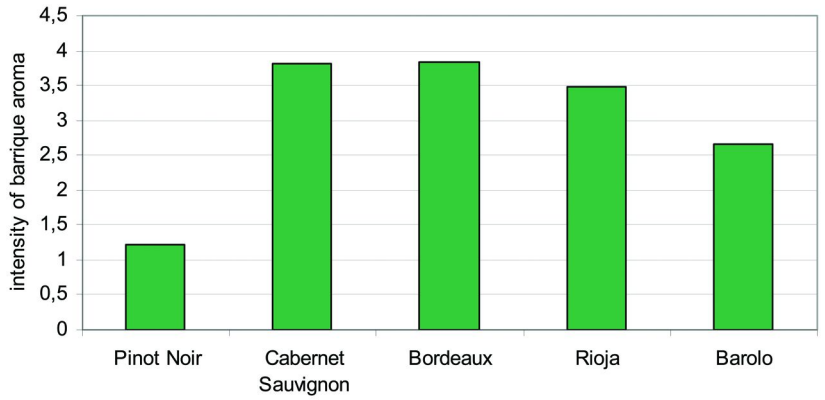

Figure 1. Intensity of the "barrique-type" aroma note in five commercial red wines.

Table III. Concentrations of cis-whiskylactone and vanillin in the five commercial red wines

Type	*Conc. (µg/L)*	
	cis-whiskylactone	*vanillin*
Pinot Noir	4.5	29
Cabernet Sauvignon	76	86
Bordeaux	200	56
Rioja	370	130
Barolo	22	62

Table IV. Odor activity values of cis-whiskylactone and vanillin in the five barrel aged wines

Type of red wine	*OAV*[a]	
	cis-whiskylactone	*vanillin*
Pinot Noir	<1	<1
Cabernet Sauvignon	1.1	1.4
Bordeaux	3.0	~1
Rioja	5.5	2.2
Barolo	<1	1.0

[a] Odor activity values were calculated by dividing the concentration (Table III) by the odor threshold of 60 µg/L for vanillin and 67 µg/L for cis-whiskylactone (*6*).

One explanation for this observation might be that, once the odor threshold has been reached, no sensorially detectable increase in the intensity might occur. To test this assumption, the following sensory study was performed: vanillin and cis-whiskylactone were individually dissolved in 13% ethanol in water in concentrations varying between 10 µg/L and 320 µg/L with doubling their concentrations in each step. In triangle tests, the concentration of one sample was doubled as compared to the two reference samples (or vice versa), and the panelists had to indicate the samples with the higher concentration. Surprisingly, for cis-whiskylactone the panel was only able to differentiate the 80 µg/L sample in expt. 3 (Table V) from the 40 µg/L sample, thus confirming the odor threshold of the lactone. A differentiation between the other reference solutions was, however, not possible. The same result was found for vanillin. The panel was only able to detect a difference in expt. 2 (Table VI), which correlates with the odor threshold of vanillin. Neither doubling the concentration up to 160 µg/L (expt. 3) nor to 320 µg/L resulted in a significant number of correct answers, i.e. an increase in the aroma intensity.

Table V. Concentrations (µg/L) of cis-whiskylactone[a] in the model solution

Expt.	*Sample A*	*Sample B*	*Sample C*	*Difference detected*
1	10	10	20	no
2	20	20	40	no
3	40	40	80	yes
4	80	80	160	no
5	160	160	320	no

[a] Odor threshold: 67 µg/L.

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Table VI. Concentrations (µg/L) of vanillin[a] in the model solution

Expt.	*Sample A*	*Sample B*	*Sample C*	*Difference detected*
1	20	20	40	no
2	40	40	80	yes
3	80	80	160	no
4	160	160	320	no

[a] Odor threshold: 60 µg/L.

These data might explain why the intensity of the *barrique*-type aroma in the Cabernet Sauvignon, the Bordeaux and the Rioja red wine were ranked with the same intensity (Figure 1), although clear differences in the concentrations of both odorants were measured (Table III). However, these results do not explain why the Barolo wine showed a clear *barrique*-type aroma (Figure 1), while cis-whiskylactone was present far below its odor activity value (Table IV) and vanillin just showed an OAV of 1. Thus, these data show that, depending on the manufacturing process, i.e. the type of oak used, further compounds may be needed to generate a *barrique*-type aroma. Since different types of oak wood are used in cooperage, either further or other compounds may contribute to the typical "*barrique*-type" odor of wines from different origins.

Conclusions

This study confirms previous results indicating that *barrique*-type aging of red wine clearly influences the intensity of the overall "woody", *barrique*-type aroma note. However, although it was shown that a *barrique* aroma can be simulated by adding five odorants derived from the oak barrel to the same wine aged in a steel tank, further studies must be undertaken to evaluate the role of oak derived aroma compounds in other red wines by systematic sensory studies in correlation with quantitative data of key aroma compounds.

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Chapter 12

Authentication of Foods Enriched with Plant Sterols/Stanols and Their Esters

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A capillary gas chromatographic (GC) method allowing the separation and the quantification of intact plant steryl and stanyl esters was developed. Using a medium polar trifluoropropylmethyl polysiloxane stationary phase, long-chain plant stanyl esters could be separated according to their stanyl moieties and their fatty acid chains. Calibrations were performed for individual esters. The GC analysis was combined with efficient isolation techniques: (i) a method allowing the rapid extraction of stanyl esters from enriched skimmed milk-drinking yogurts and (ii) on-line LC-GC enabling the pre-separation of lipid extracts from enriched margarines and the capillary gas chromatographic analysis of plant steryl/stanyl esters in a single run. The robust methods provide a basis for the authentication of these functional foods.

Introduction

Plant steryl and stanyl esters incorporated into food products are considered to be effective in reducing total and LDL cholesterol (*1–3*). In the European Union (EU), their use in foods is regulated under the Regulation (EC) No. 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients (*4*). According to this regulation, an authorization or a notification procedure is required before an enriched food product can be launched. Since the safety assessment and approval of the first food product, a yellow fat spread with phytosteryl esters, in the year 2000 (*5*),

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numerous other enriched food products, mainly margarines and low-fat carriers like milk and yogurts with phytosterols and their esters, entered the European market. The concentrations of plant sterols vary from 0.3 % in milk-type products to 8 % in spreads (*6*). For the industrial esterification, phytosterols and stanols are obtained from vegetable oils as well as from tall oil, the fatty acid moieties also from edible oils (*7*, *8*). Accordingly, enriched products were specified regarding their sterol/stanol profiles and fatty acid distributions (*5*, *9*).

At present, there are no official methods for the analysis of phytosterols/-stanols and their esters in enriched food products. There is a need for qualitative and quantitative methods allowing their authentication. Currently, analytical approaches are based on the isolation of sterols and/or their esters and the subsequent analysis by means of gas chromatography (GC) or high-performance liquid chromatography (HPLC). Commonly, the esters were subjected to alkaline hydrolysis and the liberated sterols analyzed by GC (*10–13*). In this way, information regarding the fatty acid composition of the steryl esters is lost.

Intact steryl esters were investigated by means of GC (*14–16*) or reversed-phase HPLC. For HPLC analysis, a mass spectrometric (MS) detector was required for the identification of incompletely separated as well as co-eluted steryl esters and neutral lipids (*17*). By means of GC, using non-polar stationary phases, the steryl esters were separated with respect to the carbon number, but not to the degree of unsaturation (*14*, *15*).

In this study, GC resolution of intact stanyl esters was achieved using a trifluoropropylmethyl polysiloxane-coated capillary column. This approach was combined with efficient isolation and separation techniques, enabling a rapid investigation of phytosteryl/-stanyl esters in enriched foods.

Experimental

Lipase-Catalyzed Preparation of Stanyl Esters

Fatty acid methyl esters (300 µmol) were transesterified with phytosterols/-stanols (300 µmol) in the presence of 50 mg of *C. rugosa* lipase (VII) and 500 µL *n*-hexane by magnetic stirring in a screw-capped tube at 40 °C for 72 h (*18*).

Capillary Gas Chromatography

Gas chromatographic analysis was carried out on a 30 m × 0.25 mm I.D. fused-silica capillary coated with a film thickness of 0.1 µm trifluoropropylmethyl polysiloxane (Rtx-200MS, Restek, Bad Homburg, Germany). The temperature of the injector was set to 280 °C, split flow to 11.2 mL/min, resulting in a split ratio of 1:7.5. The temperature program was 100 °C (2 min), programmed with 15 °C/min up to 310 °C (2 min), then 1.5 °C/min up to 340 °C (3 min).

For GC/FID analysis, an Agilent Technologies instrument 6890N (Böblingen, Germany) equipped with a flame ionization detector (FID) was used. Hydrogen was used as carrier gas with the constant flow rate 1.5 mL/min. The detector temperature was set to 360 °C. Nitrogen was used as make-up gas with a flow of 25 mL/min. Data acquisition was performed by ChemStation software.

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The GC/MS analysis was performed on a Finnigan Trace GC ultra equipped with a single quadrupole mass detector (DSQ; Thermo Electron Corp., Austin, TX) by electron impact ionization (EI) at 70 eV in the full scan mode at unit resolution from 40-750 Da (scan time 0.4 s). Helium was used as carrier gas with the constant flow rate 1 mL/min. The interface was heated to 320 °C, the source to 250 °C.

LC/MS

The LC/MS analysis was performed on a Dionex Ultimate 3000 RSLC Instrument coupled to a single quadrupol mass detector (MSQ Plus) (Dionex Softron GmbH, Germering, Germany). APCI was used in the positive ionisation mode in accordance with the optimized LC/MS conditions for steryl ester analysis (*17*). The probe temperature was set to 400 °C, nitrogen inlet pressure to 40 psi, the cone-voltage to 80.00 V and the corona current to 8 µA. For the separation on a Luna hexyl-phenyl HPLC column, 100 mm × 2.0 mm I.D., 3 µm particle size (Phenomenex LTD, Aschaffenburg, Germany), a MeOH/H_2O-gradient was used: from 90 to 95 % MeOH in 2 min (3 min), to 100 % MeOH in 15 min (5 min); flow rate 0.6 mL/min; The column temperature was 35 °C.

On-Line LC-GC

On-line LC-GC analysis was performed on a Dualchrom 3000 instrument (Carlo Erba Instruments, Rodano, Italy), equipped with a loop type interface (500 µL sample loop) and a fully automated interface valve system.

The HPLC pre-separation was performed on a Eurospher® -100 Si column (5 µm particle size, 250 × 2 mm I.D.) from Knauer (Berlin, Germany), thermostated to 20 °C. *n*-Hexane/MTBE (96 + 4, v + v) was used as eluent with a flow rate of 200 µL/min. LC-detection was performed at 205 nm. The backflush started directly after the transfer with 200 µL/min MTBE for 7 min.

The transfer start was set 5.15 min after the injection. An early solvent vapor exit was opened during the transfer and closed automatically 60 sec after a pressure reduction of 80 kPa.

The GC separation was performed on a Rtx-200MS, 27 m × 0.25 mm I.D., 0.1 µm d_f (Restek, Bad Homburg, Germany) connected in series with an uncoated capillary deactivated with DPTMDS (3 m × 0.53 mm I.D., BGB Analytics Vertrieb, Schloßböckelheim, Germany) and a coated precolumn (3 m x 0.25 mm I.D. 0.1 µm d_f Rtx-200MS). Hydrogen was used as the carrier gas with a constant flow rate of 1.7 mL/min. After holding the transfer temperature of 140 °C for 5 min, the temperature was programmed with 15 °C/min up to 310 °C (2 min), then 1.5 °C/min up to 340 °C (3 min). The FID temperature was set to 360 °C. Nitrogen was used as make-up gas with a pressure of 120 kPa. All components were controlled by the Dualchrom software. The Chromcard software was used for data collection and processing.

Results and Discussion

Capillary-Gas Chromatographic Separation and Quantification of Long Chain Fatty Acid Plant Steryl/Stanyl Esters

Phytosteryl and stanyl long chain fatty acid esters were prepared by enzyme-catalyzed transesterification of fatty acid methyl esters using *C. rugosa* lipase as biocatalyst (*18*).

Molecular ions $[M]^+$ of low intensity were obtained for all stanyl esters and for steryl esters of polyunsaturated fatty acids. Spectra of steryl esters as well as the saturated plant stanyl esters exhibited $[M\text{-}FA]^+$ as base fragment, esters of unsaturated fatty acid moieties $[M\text{-}FA+H]^+$. The characteristic fragment ions reported for steryl and stanyl acetates (*19*, *20*) were present in all of the spectra of synthesized long chain fatty acid esters. As examples, the mass spectra of ß-sitosteryl and sitostanyl linolenate are shown in Figure 1.

The capillary gas chromatographic separation of long-chain fatty acid esters of stanols was achieved using an intermediately polar trifluoropropylmethyl polysiloxane (Rtx-200MS) stationary phase. The analysis of an enzymatically synthesized plant stanyl ester mixture is shown in Figure 2A; only saturated and mono-unsaturated fatty acid esters of the same carbon number eluted at the same time. Sufficient separation was achieved for esters with unsaturated fatty acid moieties and for esters of different carbon numbers. In comparison, analysis of this mixture by LC did not result in a complete resolution of the campestanyl and sitostanyl esters (Figure 2B).

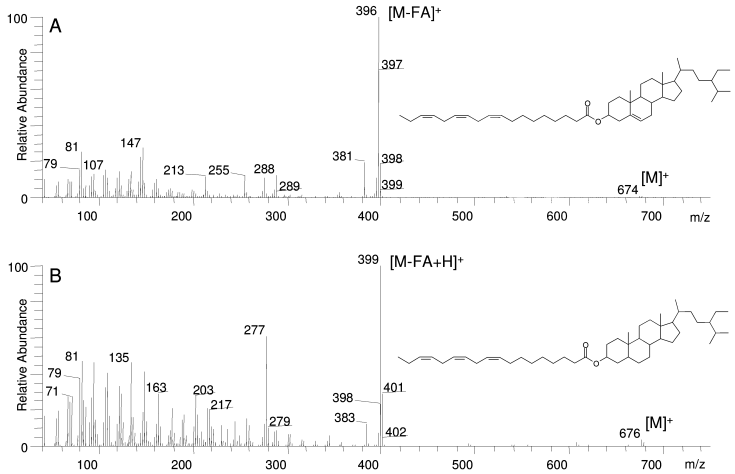

Figure 1. Electron-impact ionization mass spectra of ß-sitosteryl (A) and sitostanyl linolenate (B); $[M]^+$: molecular ion, [FA]: fatty acid, [H]: hydrogen.

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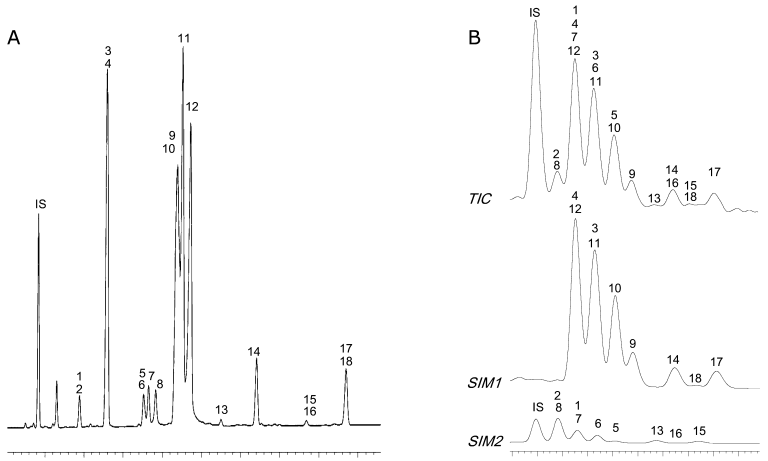


Figure 2. Chromatographic separation of a mixture of synthesized plant stanyl esters by means of GC (A) and RP-LC/MS (B); Peak identification: (1) campestanyl 16:0, (2) campestanyl 16:1, (3) sitostanyl 16:0, (4) sitostanyl 16:1, (5) campestanyl 18:0, (6) campestanyl 18:1, (7) campestanyl 18:2, (8) campestanyl 18:3, (9) sitostanyl 18:0, (10) sitostanyl 18:1, (11) sitostanyl 18:2, (12) sitostanyl 18:3, (13) campestanyl 20:0, (14) sitostanyl 20:0, (15) campestanyl 22:0, (16) campestanyl 22:1, (17) sitostanyl 22:0, (18) sitostanyl 22:1, (IS) cholesteryl 16:0; total ion current (TIC); selected ion monitoring of sitostanyl esters (SIM1, m/z 399-400) and campestanyl esters (SIM2, m/z 385-386).

LC/MS was performed on a hexyl-phenyl stationary phase, previously employed for the analysis of plant steryl esters (*17*). Sitostanyl and campestanyl esters were distinguishable by selected ion monitoring (SIM), according to their base fragment $[M\text{-}FA+H]^+$ at *m/z* 399-400 (SIM1) and *m/z* 385-386 (SIM2), respectively. However, the resolution of the individual stanyl fatty acid esters was still unsatisfactory. For example, the esters of palmitic acid and linoleic acid eluted at the same time.

The thermal instability of the high boiling steryl esters during GC analysis is a well known issue (*21*, *22*). In order to compensate for the degradational losses, response factors were determined. As individual substances were not commercially available as references, the degree of degradation of plant steryl and stanyl esters under the employed gas chromatographic conditions was studied with industrial phytosteryl/stanyl ester mixtures and determined for the Δ^5-steryl and stanyl fatty acid ester groups, respectively. As shown in Table I, the degree of degradation increased with the number of double bonds and with the time of elution from the column. On the basis of these data, calibration curves allowing the accurate quantification of individual esters were established. The detector response was linear in a concentration range from 0.1 to 0.5 µg total phytosteryl/-stanyl esters / µL injection volume.

Table I. Response factors determined for plant steryl and stanyl ester analysis by GC/FID

	response factor[a]	
esterified fatty acid	*Δ⁵-steryl esters*[b]	*stanyl esters*[c]
16:0/16:1	1.02	0.94
18:0/18:1/18:2	1.29	1.16
18:3	-	1.68
20:0/20:1	-	1.55
22:0/22:1	-	2.86

[a] referring to the internal standard cholesteryl palmitate. [b] determined with Vegapure® 95E (Cognis GmbH, Illertissen, Germany). [c] determined with STAEST-115 (Raisio Group, Raisio, Finland).

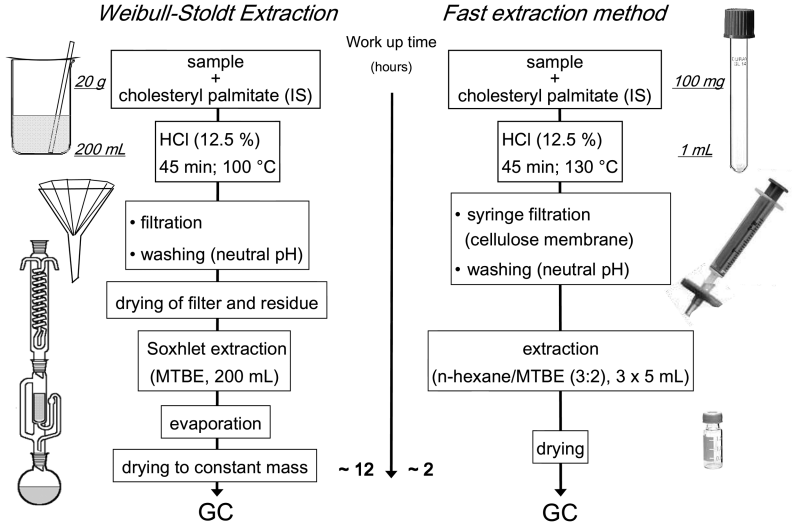
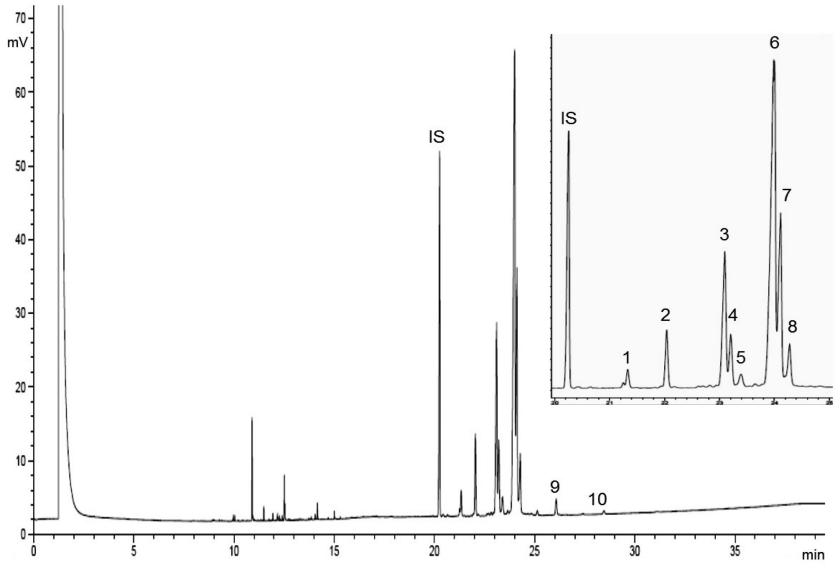


Figure 3. Comparison of the work-up procedures according to the Weibull-Stoldt extraction and the fast extraction–method.

Investigation of Plant Stanyl Esters in Skimmed Milk-Drinking Yogurts

Skimmed milk-drinking yogurts were selected to demonstrate the applicability of the GC-based approach. The advantage of this type of product is the lack of an interfering lipid matrix. On the other hand, the protein content of these products may hamper the quantitative extraction of the lipids. Therefore, the initial sample preparations were carried out by employing the extraction according to Weibull-Stoldt (*23*). A "digestion" under acidic conditions is performed before the extraction of the lipids. However, the sample preparation according to this method requires about 12 h. To accelerate the procedure and

to render the approach more appropriate for routine analysis, the fast extraction method depicted in Figure 3 was established. This involved a reduction of the sample size and of the volume of acid employed for digestion, a filtration via syringe filter and the extraction with smaller amounts of solvent rather than in a Soxhlet apparatus. With these modifications the lipid extraction can be performed within two hours in triplicate (*24*). For plant stanyl esters (STAEST-115 mixture) added to a skimmed milk-yogurt recoveries of 100 ± 1 % were determined.



plant stanyl esters	*[g/100g]*
(1) campestanyl-16:0/16:1	0.07 ± 0.00 [a]
(2) sitostanyl-16:0/16:1	0.19 ± 0.00
(3) campestanyl-18:0/18:1	0.78 ± 0.01
(4) campestanyl-18:2	0.26 ± 0.00
(5) campestanyl-18:3	0.11 ± 0.00
(6) sitostanyl-18:0/18:1	2.41 ± 0.04
(7) sitostanyl-18:2	0.81 ± 0.04
(8) sitostanyl-18:3	0.28 ± 0.01
(9) sitostanyl-20:0/20:1	0.07 ± 0.00
(10) sitostanyl-22:0/22:1	0.02 ± 0.00
others	0.07 ± 0.01
total esters	5.07 ± 0.07

esterified stanols [g/100g][b]	*3.1 ± 0.1*
campestanol [%]	24.5 ± 0.3
sitostanol [%]	75.5 ± 0.3
esterified fatty acids [%][b]	
C16:0 / C16:1	5.2 ± 0.1
C18:0 / C18:1	63.8 ± 0.5
C18:2	21.5 ± 0.5
C18:3	7.6 ± 0.2
C20:0 / C20:1	1.5 ± 0.0
C22:0 / C22:1	0.4 ± 0.2

[a] values represent averages ± standard deviations of 18 analyses
[b] calculated on the basis of phytosteryl esters

Figure 4. Gas chromatographic analysis of stanyl esters extracted from a commercial enriched skimmed milk-drinking yogurt; each of the six bottles contained in a package was analyzed in triplicate.

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The capillary gas chromatographic separation and the quantification of the stanyl esters isolated from an enriched skimmed milk-drinking yogurt (Emmi-Benecol®) are presented in Figure 4. The spectrum comprised sitostanyl and campestanyl derivatives, with sitostanyl C18-esters as predominating fraction. A total ester content of 5.07 ± 0.07 g/100g was determined. The amount of esterified stanol (3.1 ± 0.1 g/100g), calculated from the ester distributions, was in good agreement with the declaration of the product ("plant stanol 3.0 %"). The proportions of the esterified fatty acid moieties indicated rapeseed oil as fatty acids source. The standard deviations of the analytical results obtained were very low, demonstrating the good repeatability of the developed quantification approach.

Using the fast extraction method, the phytosteryl/-stanyl ester preparations were investigated in several enriched drinking yogurts purchased in German and Italian supermarkets. Figure 5 shows examples of the variability observed for the profiles of added phytosteryl/-stanyl esters. The identities of the esters were confirmed using enzymatically synthesized reference substances by comparison of their retention times and mass spectra. Besides the expected mixtures of long chain fatty acid phytosteryl/-stanyl esters, medium chain fatty acid esters were revealed as ingredients in one of the investigated products (*25*). As indicated in Figure 5B, the plant steryl esters were baseline-resolved from their respective stanyl esters under the employed capillary gas chromatographic conditions.

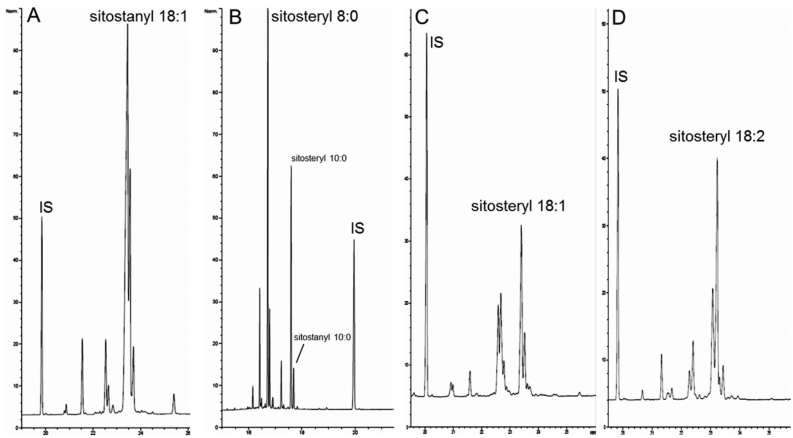

Figure 5. Examples of phytosteryl-/stanyl ester mixtures added to drinking yogurts commercially available in the EU; (A) stanyl esters of rapeseed oil fatty acids (Emmi-Benecol® (Germany), 3.0 % plant stanols); (B) steryl/stanyl esters of medium chain fatty acids (Danacol® (Italy), 1.6 % phytosterols); (C) steryl/stanyl esters of rapeseed oil fatty acids (Alpina® (Italy), 1.6 % phytosterols); (D) steryl/stanyl esters of sunflower oil fatty acids (Becel pro activ® (Germany), 2.0 % phytosterols).

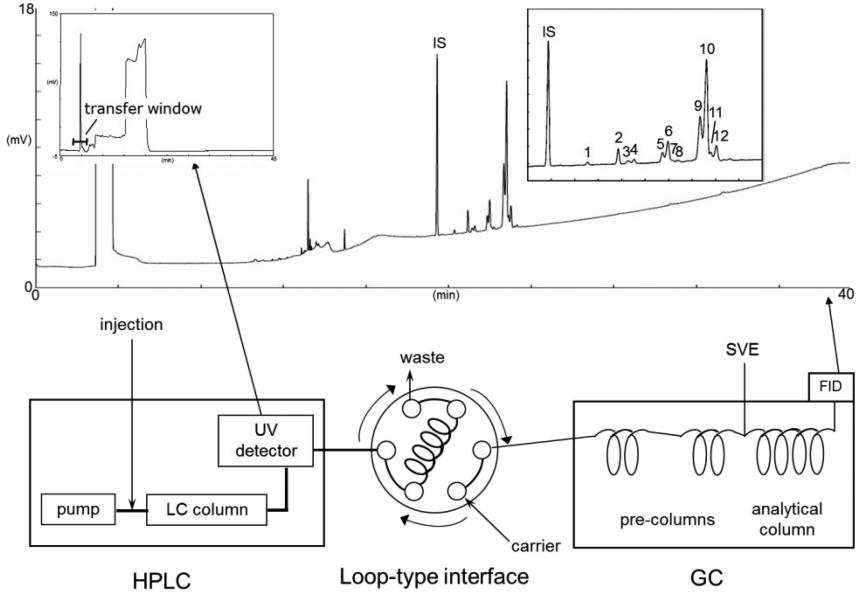

Figure 6. Analysis of phytosteryl/-stanyl fatty acid esters in enriched spread by means of on-line LC-GC (peak numbering according to Table II).

Investigation of Plant Steryl/Stanyl Esters in Enriched Margarine by On-Line LC-GC

Enriched margarines were selected as second type of product. The acid digestion step is not necessary for the extraction of the lipids from margarines, but the GC analysis of phytosteryl esters in products with such high fat contents requires a pre-separation of the esters from disturbing lipids, like di- and triglycerides. The isolation of the esters was performed by normal-phase HPLC: the first eluting fraction consisted of the steryl esters. The isolation of the esters was performed by HPLC. By on-line coupling of LC and GC via a loop type interface (Figure 6) the ester fraction was transferred directly into the GC system. This procedure allows the isolation of the esters and the capillary gas chromatographic separation in a single run.

The list of ingredients on the package of the investigated enriched margarine (Becel pro activ®), purchased from a local supermarket, included the following information: "12.5 % phytosteryl esters, according to 7.5 g phytosterols per 100 g; 40 % fat". The results obtained by the on-line LC-GC investigation of the lipid extracts are given in Table II. Sitosteryl-C18 esters were determined as the predominating fraction; the minor amounts of stigmasteryl esters co-eluting with the respective campesteryl esters (peak 6) and campestanyl esters (peak 7) were negligible regarding the overall quantification. The contents of esters co-eluting in peak 3 were calculated on the basis of the ester distribution in the mixture used for calibration. The quantified esters as well as the calculated esterified phytosterol content agreed well with the package declaration. The calculated

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profile of esterified sterols was in accordance with the specification of the SCF (*9*). The distribution of esterified fatty acids indicated sunflower seed oil as fatty acid source.

In summary, the developed approaches are suitable for fast and efficient analyses of products with added phytosteryl/stanyl esters by means of capillary gas chromatography. The methods provide information on the identities and contents of individual esters and thus offer a basis for the authenticity assessment of this type of enriched foods.

Table II. On-line LC-GC analysis of phytosteryl/-stanyl esters in enriched margarine (Becel pro activ®)[a]

phytosteryl esters	*[g/100g]*
(1) campesteryl-16:0/16:1	0.09 ± 0.00[b]
(2) sitosteryl-16:0/16:1	0.52 ± 0.02
(3) sitostanyl-16:0/16:1 + brassicasteryl-18:0/18:1	0.16 ± 0.00
(4) brassicasteryl-18:2	0.20 ± 0.01
(5) campesteryl-18:0/18:1	0.51 ± 0.06
(6) campesteryl-18:2 + stigmasteryl-18:0/18:1	1.28 ± 0.03
(7) campestanyl-18:0/18:1 +stigmasteryl-18:2	0.06 ± 0.02
(8) campestanyl-18:2	0.06 ± 0.01
(9) sitosteryl-18:0/18:1	2.66 ± 0.09
(10) sitosteryl-18:2	5.89 ± 0.14
(11) sitostanyl-18:0/18:1	0.34 ± 0.04
(12) sitostanyl-18:2	0.64 ± 0.02
others	0.10 ± 0.01
total esters	12.50 ± 0.24

esterified sterols [g/100g][c]	7.6 ± 0.1
brassicasterol [%]	2.4 ± 0.1
campesterol [%]	15.1 ± 0.3
stigmasterol [%]	n.c.
campestanol [%]	1.0 ± 0.3
sitosterol [%]	72.5 ± 0.7
sitostanol [%]	8.3 ± 0.4
others [%]	0.8 ± 0.1
esterified fatty acids [%][c]	
C16:0 / C16:1	5.3 ± 0.2
C18:0 / C18:1	29.4 ± 0.1
C18:2	64.6 ± 0.2

[a] one package was worked up in triplicate

[b] values represent averages ± standard deviations

[c] calculated on the basis of phytosteryl esters

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Chapter 13

Authenticity Control of Oils and Fats via Large Capacity Sorptive Extraction

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Advanced tools for the rapid identity check and authenticity control for complex flavor raw materials as well as the analysis of contaminants is of increasing importance for food and flavor companies. The mega trend for natural ingredients requires an optimized combination of effective sample clean-up and target analysis for key compounds. In this context modern adsorption technologies are providing selectivity adjusted analytical work procedures for direct analysis of difficult matrices such as dairy products and other fat and oil containing raw materials as well as complex juice derived materials.

For this purpose the polymer adsorption based Large Capacity Sorptive Extraction (LCSE) technology offers a rapid access to a broad spectrum of flavor and taste active materials. The combination of LCSE technology with other analytical methodologies such as LC-MS provides a comprehensive picture of the identity and authenticity of raw materials with a parallel insight into the sensorial performance in the final application.

This approach is of particular interest for the analysis of oils and lipids, which are often showing difficulties in flavor analysis because of the limited recovery rate for specific aroma active substances and corresponding matrix effects.

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Introduction

The analytical and culinary authenticity of olive oils is associated with genetic variety, geographical origin and quality grade. The European legislation (*1*) basically differentiates between the following categories of virgin olive oil: extra virgin olive oil (EVOO), virgin olive oil (VOO), lampante virgin olive oil (LVOO) (*2*). In addition, refined olive oil (ROO) and olive oil, which is a mixture of virgin olive oil and refined olive oil are characterized. A second category of olive oil is obtained from olive pomace via extraction and needs to be labelled crude olive pomace oil (COPO) and olive pomace oil (OPO), which is a mixture of refined olive pomace oil and virgin olive oil. Additional standards are provided by the Codex Alimentarius Commission (*3*) and the International Olive Oil Council (*4*). This highly complex regulatory situation requires a broad set of analytical parameters in order to support the authentication procedure for precious oils and fats and in particular for olive oils.

Analytical Tools

In the last 20 years a broad portfolio of analytical tools for the authenticity control and the correct classification of olive oils has been developed. In 2007, Fritsche and Hrncirik developed a decision tree to determine the conformity of extra virgin olive oils with the respective quality criteria (*5*).

In this decision tree the following main selection criteria were used: acidity, peroxide value, UV spectrometry and organoleptic assessment. Fritsche and Hrncirik (*5*) also proposed a second decision tree for the conformity of extra virgin or virgin olive oil with the respective purity criteria. Main selection criteria are the content of 3,5-stigmadienes, the content of trans isomers, the match of the fatty acid profile, the match of the sterol profile, the presence of erythrodiol and uvaol as well as the wax content. In many cases stigmasta-3,5-diene is considered as a main indicator compound for the presence of refined oils. This compound is formed by dehydration of ß-sitosterol during the bleaching step and shows a linear correlation with the amount of bleaching earth. The analysis of sterols and their respective dehydration products is a challenge for conventional GC analysis techniques. Due to their low volatility and the potential co-elution with high amounts of squalene analytical difficulties need to be solved. Some of the published analytical techniques like the coupling of LC with GC address these issues (*6*). Coupled LC-GC instruments, however, do represent a highly specialized equipment which is not readily available in many analytical laboratories. Moreover, high injection volumes from the LC instrument into GC injector are often influencing the performance of the analytical separation. An alternative route is represented by the extraction of the unsaponifiable matter, subsequent fractionation of the sterols on silica gel, followed by a detailed GC analysis. Recently, mass spectrometric methods have been published which allow the analytical detection of sterols and link their quantities with the authenticity or even the detection of monovarietal virgin olive oils (*7*, *8*). The detection of the two hydroxytriterpenes erythrodiol (3ß,28-dihydroxy olean-12-en) and uvaol (3ß,28-dihydroxy urs-12-en) can be achieved with the methods discussed before

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and represents a parameter for the detection of olive pomace oils. Uvaol and erythrodiol are present in olive skins and are extracted during solvent extraction or second extraction steps and consequently occur at higher levels in pomace oils or oils from a second pressing.

Fritsche and Hrncirik (*5*), also highlight the importance of minor components such as phenols. These trace constituents contribute to the unique taste of virgin olive oil. Main constituents of this phenolic fraction are represented by phenols, secoiridoid aglycones, lignans, flavonoids and other natural products and provide a valuable link between analytical and culinary authenticity.

The characteristic flavor of olive oil depends on the cultivar, growing conditions (climate, soil, etc.), ripeness and processing of the oils. In some cases, virgin olive oils of lower quality show a more complex profile due to additional volatiles formed by oxidation during storage or by the activity of bacteria and moulds. The analysis of aroma compounds from oily matrices is usually performed via dynamic headspace stripping (*9*), simultaneous distillation extraction SDE after Likens-Nickerson (*10*), high-vacuum stripping (*11*) or Solid Phase Micro Extraction (SPME).

All described analytical parameters require a broad spectrum of established methods, some of which are quite complex and are subject for reduced reproducibility because of the highly lipophilic oil matrix. In this situation large capacity sorptive extraction (LCSE) with increased polymer polarity is able to provide the right selectivity for the analytes of interest. In this study the flavor profiles of different olive oils were recorded using a self-prepared vinyl based polymer. In addition an olive oil spiked with 10% hazelnut oil was analyzed using LCSE as a flavor extraction technique. For a comprehensive analytical and sensory profile of the individual oil qualities the GC-MS data of volatiles as well as LC-MS data of phenolic compounds provides an additional marker profile for the assessment of extra virgin olive oils.

Results

In general solvent extraction as well as sorptive extraction techniques based on unpolar polymers show significant co-extraction of an oil matrix. This is especially known for polydimethyl siloxane based polymers (PDMS) which are used for a commercial stir bar sorptive extraction (SBSE) tool and are also known as Twister® (*12*). The direct thermo desorption (TDS) of oils in TDS tubes is an alternative route. For this approach, a careful selection of the TDS temperature is required.

In this study a newly developed sorptive extraction technique based on a polymer with higher polarity compared to PDMS and a glass stick based sampling tool with larger capacity compared to the commercially available Twister® was used. Based on these properties, the extraction technique will be referred to as Large Scale Sorptive Extraction (LCSE) in the following text. As a first step for the validation of the LCSE technique the recovery of aroma compounds from oil matrices, the reproducibility and the robustness was investigated.

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Table 1. Recovery rates [%] from water and oil obtained by three different techniques relative to a direct injection of a compound mixture in solvent

	SAFE		SBSE		LCSE	
	water	oil	water	oil	water	oil
ETHYLBUTYRATE	30,6	52,3	2,6	n.d.	16,8	0,7
PINENE, BETA-	n.d.	44,5	18,3	n.d.	20,2	n.d.
LIMONENE	n.d.	41,8	17,5	n.d.	31,6	0,4
HEXANALDIETHYLACETAL	30,7	20,6	12,3	n.d.	9,0	n.d.
FILBERTONE	53,1	34,4	4,2	n.d.	13,8	0,3
2,3-DIMETHYLPYRAZINE	66,0	36,1	0,2	n.d.	1,9	0,5
LINALOOL	67,1	12,0	2,8	n.d.	11,9	0,2
GERANIOL	72,4	n.d.	4,2	n.d.	11,8	0,5
DECALACTONE, GAMMA-	77,5	n.d.	6,9	n.d.	17,9	0,4
CYCLOTENE	58,6	n.d.	n.d.	n.d.	0,6	0,7
CAPROIC ACID	n.d.	n.d.	10,8	n.d.	20,8	9,4
HELIOTROPIN	132,4	n.d.	1,5	n.d.	7,0	0,5
LAURIC ACID	n.d.	n.d.	2,6	n.d.	38,7	0,7
FURANEOL	n.d.	n.d.	n.d.	n.d.	0,5	0,2
SULFUROL	31,1	n.d.	0,1	n.d.	0,8	0,5
CUMARIN	73,1	n.d.	0,7	n.d.	7,1	0,5
VANILLIN	86,2	n.d.	0,1	n.d.	0,9	0,3
ACETIC ACID	n.d.	n.d.	0,7	0,1	6,5	7,5

For the direct measurement of the recovery rate a comparison experiment by TDS with 3 different sampling techniques was performed: Solvent Assisted Flavor Evaporation SAFE, SBSE and LCSE (*13*). A solution of 0.1 ppm of individual aroma compounds diluted in a suitable solvent was directly introduced onto silanized glass wool into a TDS tube, thermo-desorbed and peak areas recorded by GC-MS. The same compounds were dissolved at an equal concentration of 0.1 ppm in either oil or water. These solutions have then been extracted with the described 3 techniques SAFE, SBSE and LCSE. The resulting extracts have again been introduced into TDS tubes and again peak areas have been recorded by GC-MS. Table 1 shows the results. Peak areas from the solution in solvent were set to 100%. Peak areas from the sorptive extractions were calculated relative to these figures.

The data shown in Table 1 clearly indicate an improved extraction of molecules with higher polarity from oil matrices like e.g. 4-Hydroxy-2,5-dimethyl-3(2H)-furanone (Furaneol®) or vanillin.

The robustness of the LCSE technique was also investigated using matrices with different fat contents. Increasing fat content suppresses the extraction rate of volatile compounds to a very different extend. This has been tested by adding a test mixture to milk and cream with increasing fat content and subsequent calculation of the recovery rates in a previous study. As a consequence the recovery rates of the analytes of interest have to be determined on the matrix of the sample to be analyzed. In addition competitive, sorptive effects on the polymer at

high concentrations have to be considered. The recovery of test compounds from yoghurt vs. milk illustrates this effect. Yoghurt contains more matrix compounds such as high levels of organic acids which presumably compete with the flavor molecules on the polymer. Figure 1 shows that the recovery of aroma compounds is higher in milk than in yoghurt due to the competitive sorptive effects.

In the development of analytical methods based on sorptive techniques the concentration/response function is of high importance and was analyzed using a series of dilution experiments with peak recording. The response was recorded in relation to the compound concentration in the respective matrix. Figure 2 shows 3 examples which were obtained in dilution experiments of a red wine sample. The response curves are structure-dependent. The volatility and polarity of the compounds are key factors determining the function of the response curves. Compounds with log POW values below 2 showed a polynomic behavior, whereas response curves for compounds with log P_{OW} values above 2 are linear in nature. For a comprehensive method development and subsequent method validation all previously effects were found to be highly relevant. The results of this study show that LCSE is a reproducible technique. In addition the recovery of especially polar compounds from oil is significantly improved compared to the SBSE using PDMS polymers and SAFE under standard conditions.

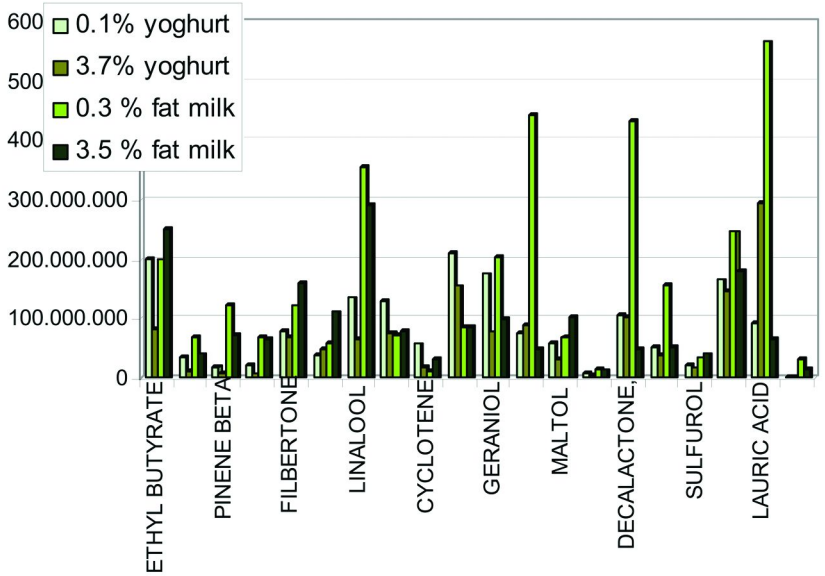

Figure 1. Extraction rate of selected test compounds added to milk and yoghurt with different fat contents.

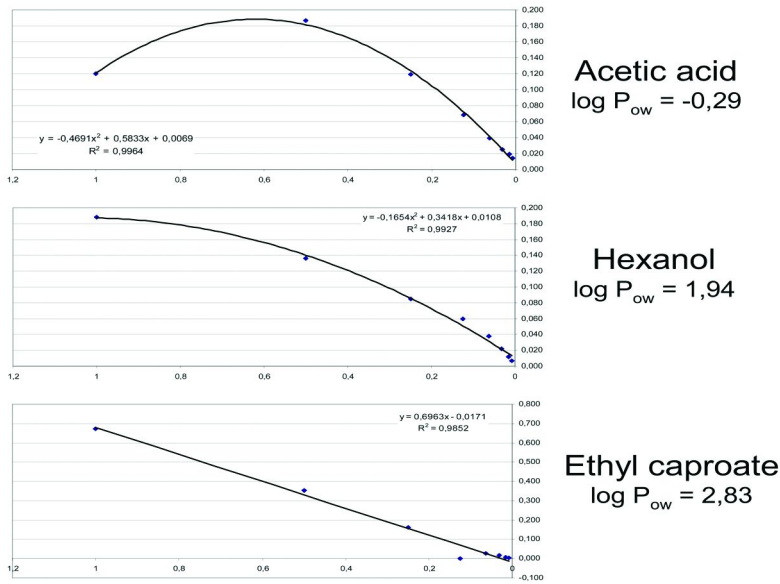

Figure 2. Response curves of acetic acid, hexanol and ethyl caproate at various dilutions of red wine (0,5; 0.25; 0.125; 0,0625).

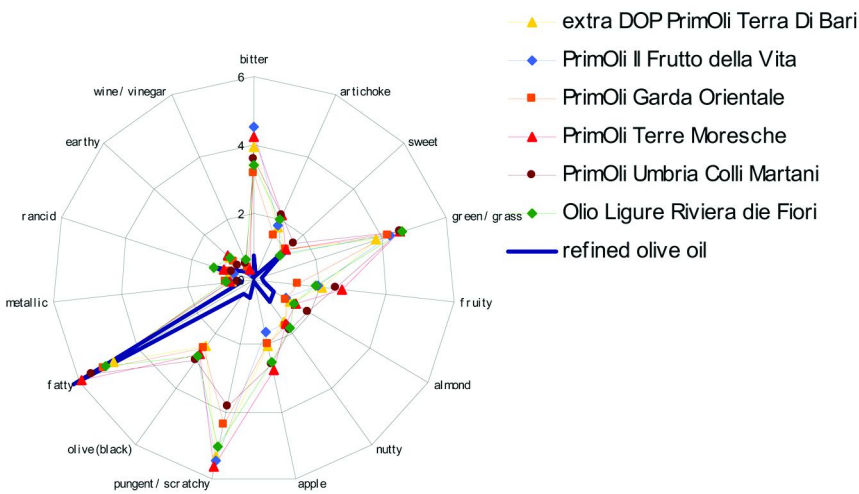

Figure 3. Sensorial profiling of 6 different virgin olive oil qualities from Italy purchased in a local supermarket. The dark blue line respresents the sensorial profile of a refined olive oil.

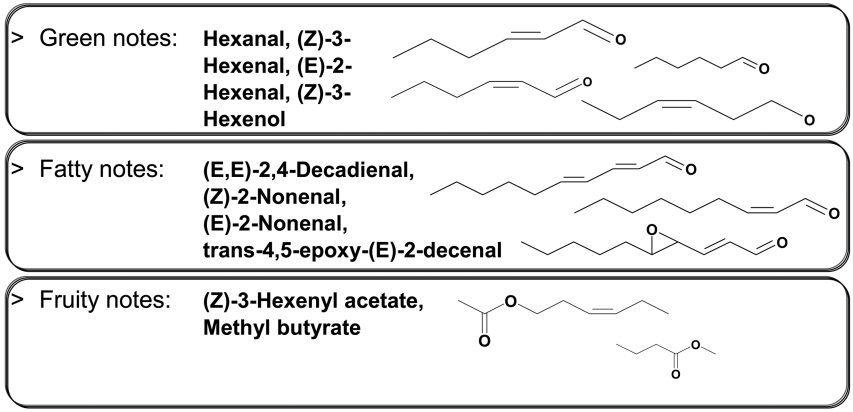

Figure 4. Most potent aroma compounds in virgin olive oils (11).

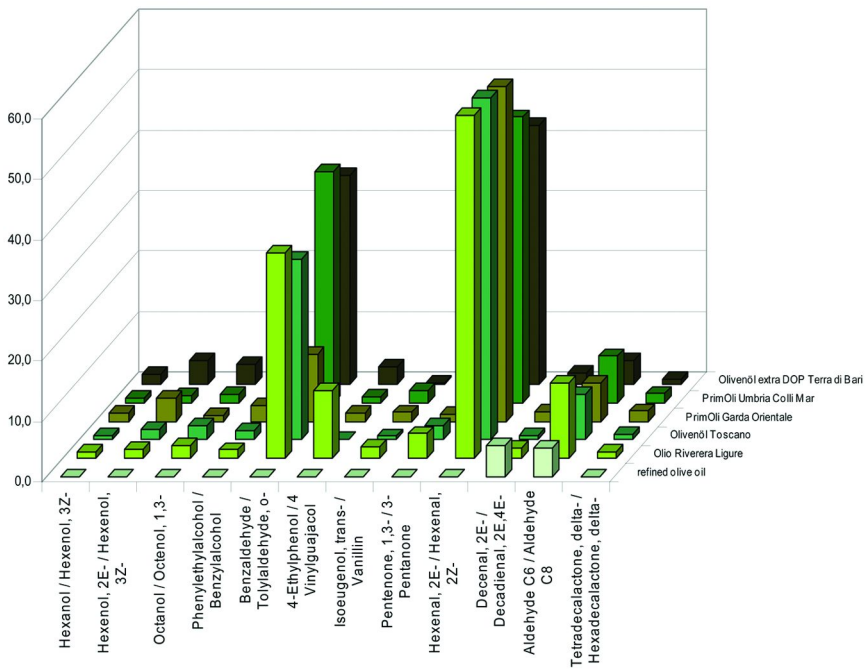

Figure 5. Aroma patterns of different extra virgin olive oils in comparison to a refined olive oil.

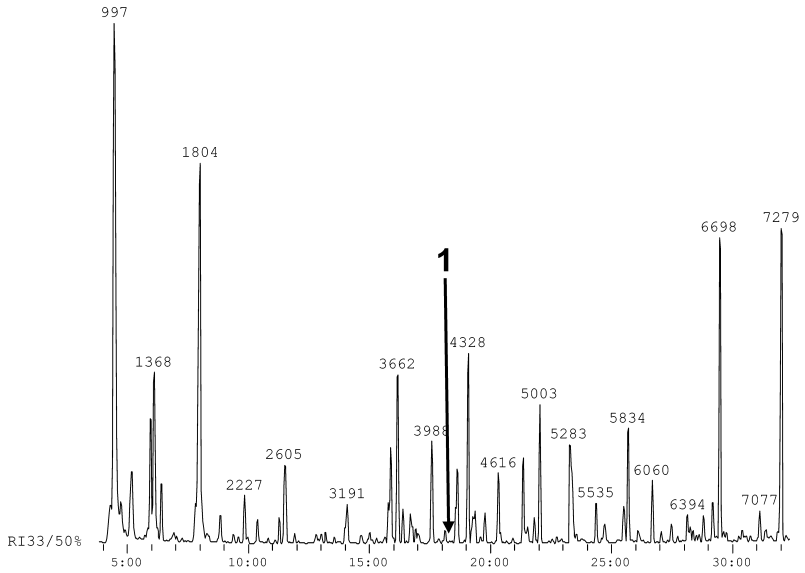

Figure 6. Filbertone determination in a 9/1 mixture of olive oil and hazelnut oil. Filbertone peak highlighted (bold 1).

In a next step LCSE was applied to the direct analysis of olive oils. Several extra virgin olive oils from various regions in Italy have been screened by a trained sensory panel using the descriptors shown in Figure 3. The main differences in the sensorial profile have been found in green, fruity and 'throat-burning' pungent and irritant notes.

The most potent aroma molecules are displayed in Figure 4.

Defined aroma profiles were obtained from 6 extra virgin olive oil samples from Itlay (PrimOli Il Frutto Della Vita, PrimOli Garda Orientale, PrimeOli Umbria Colli Martani, extra DOP PrimOli Terra di Bari, PrimOli Terre Moresche, Olio Ligure Riviera dei Fiori) and 1 sample of refined oil using LCSE. The data obtained from the refined oil sample show significantly reduced values of the individual flavour compounds. Figure 5 shows that ratios calculated from relevant aroma compounds are in relatively narrow range for extra virgin oils. The selected refined oil, however, does not fit in the described pattern.

In the literature the adulteration of olive oil with hazelnut oil is a frequently reported fraud which can be detected via the presence of characteristic aroma compound of hazelnuts like for example filbertone, 5-Methyl-2(*E*)-hepten-4-one (*14*, *15*). In this study a mixture of extra virgin olive oil (90%) and hazelnut oil (10%) was analyzed. Filbertone was identified based on LCSE-GC-MS data at trace level (1-2 ppb) analyzed via full scan GC-MS in EI mode as shown in Figure 6.

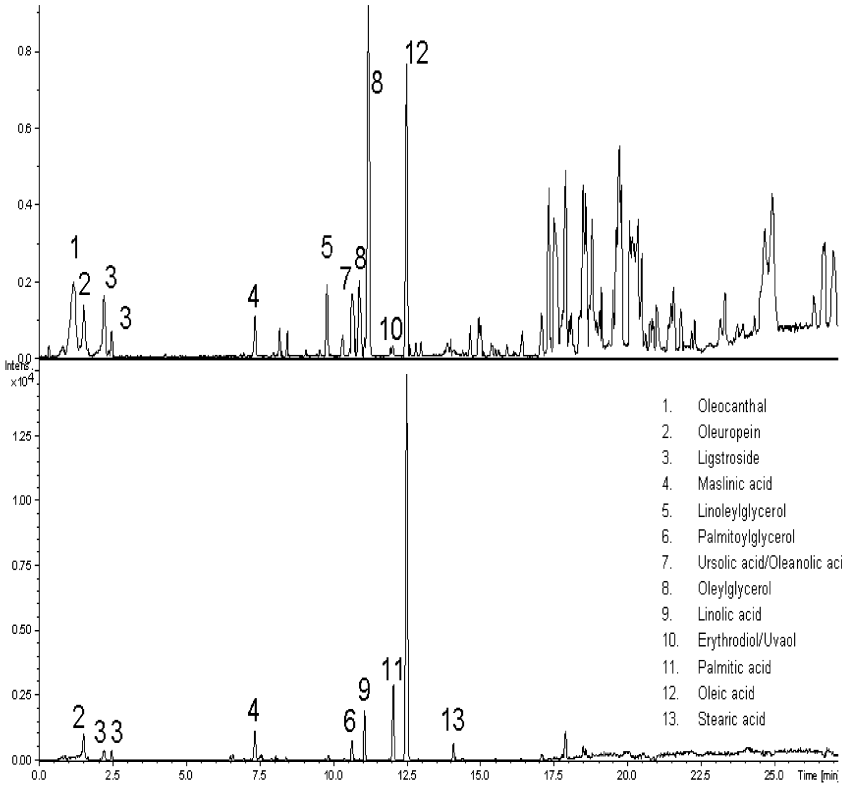

Figure 7. LC-MS chromatogram of extra virgin olive oil recorded on an UPLC-QTOF instrument both in positive and negative ionization modes.

Modern LC-MS technologies like for example Ultra Performance Liquid Chromatography – Quadrupole Time of Flight (UPLC-QTOF) systems provide an additional information including the precise moleceluar weight mass for the authentication and quality control of olive oils. Extensive spectra libraries help to facilitate the peak picking and alignment process. The parallel measurement of olive oil in both positive and negative ionization mode delivers an overview about the full spectrum of non-volatile compounds in one run. Figure 7 shows a LC-MS chromatogram of a representative extra virgin olive oil.

The pattern of non-volatiles is depending on olive tree cultivars and the corresponding growing conditions. In refined oils the quantitiative pattern of phenolic compounds is significantly reduced compared to extra virgin qualities (Figure 8). In addition the phenolic fingerprint provides a valuable correlation to the sensorial properties of the oils. Some of the phenolic compounds are also known to have potential beneficial effects on human health (*16–18*). The application of chemometric calculations with such data might even allow the monovarietal classification (*7*).

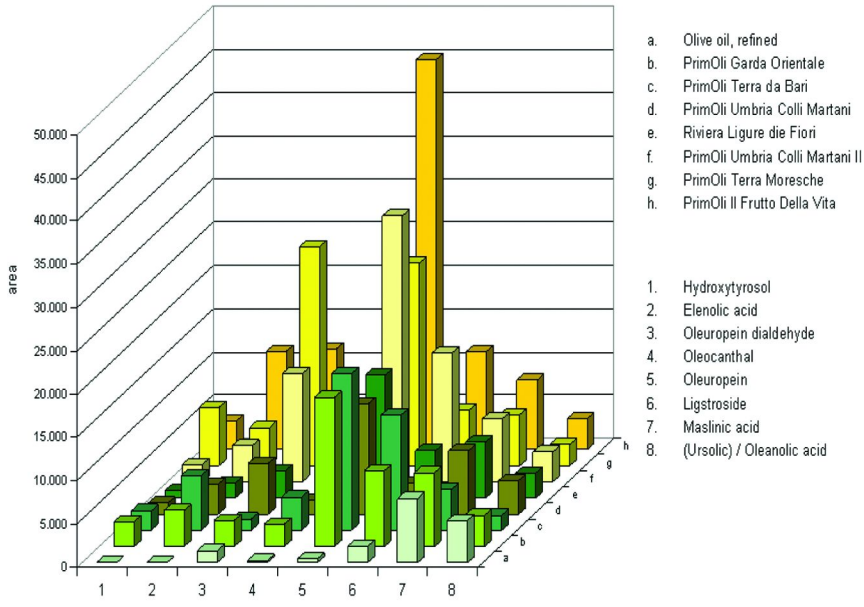

Figure 8. Non-volatile pattern of different extra virgin olive oils and a refined oil obtained by peak area comparisons resulting from UPLC-QTOF measurements.

Conclusions

The analytical authentication of olive oils requires the determination of a broad spectrum of analytical parameters. The newly developed Large Capacity Sorptive Extraction technique (LCSE, also known as SymStixx®) is based on polar polymers with a specially treated glass stick carrier and provides a rapid access to a broad spectrum of analytes in extra virgin and refined olive oils via the coupling of thermodesorption sampling and GC-MS (TDS-GC-MS). In addition LCSE analysis comprises a broad spectrum of volatile flavor active substances which helps to correlate analytical data and sensory profiles which is of upmost importance for the culinary authenticity of precious oil qualities.

Experimental Section

For GC-MS measurements an Agilent 6890 plus (Waldbronn, Germany), was used with an MSD 5973N, enhanced chemstation MSD D.03.00.611, TDS A: Gerstel (Mühlheim an der Ruhr, Germany), Maestro Vers. 1.2.5.23/3.2, conditions: TDS: 20°C ramped 60°C/min, end temperature 230°C, hold time 5 minutes, splitless mode, CIS4: -150°C ramped 12°C/sec, end temperature 230°C, hold time 5 minutes, splitless (solvent venting) 5 minutes, vent. flow 40mL/min, column: ZB-Wax+ (Phenomenex, Aschaffenburg, Germany), 60m length, 0.32mm i.d., 0,25µm film thickness, run conditions: 40°C hold for 2 minutes, ramped 4°C/min, end temperature 240°C, const. flow 2.2mL/min,

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splitless mode; MS: mass range: m/z 25 – 400, ion source: 230°C, quadrupole: 150°C, EI energy: 70 eV.

For LC-MS, a Bruker MicroQTOF II (Bremen, Germany) was used, coupled to a Waters UPLC. Gradients were run on a Phenomenex Kinetex C18 1.7 µm column (dimensions 100 x 2.1 mm) and were run from 70% H_2O with 0.1% formic acid / 30% acetonitrile with 0.1% formic acid to 0% H_2O with 0.1% formic acid / 100% acetonitrile with 0.1% formic acid in 20 minutes, holding it for further 10 minutes. Flow was at 0.55 mL/ min. Column temperature was at 50°C. DAD was measuring from 190 to 800 nm. MS was operating in ESI (Electrospray ionization) positive mode from 70 to 1600 amu for MS and MS/MS spectra.

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Chapter 14

Phenolic Compounds as Markers for the Authentication of Sherry Vinegars: A Foresight for High Quality Vinegars Characterization

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Sherry vinegars are appreciated products reaching higher prices in the market than Sherry wine. Actually, the Protection of Designations of Origin is recognized since 1995. Their elaboration encompasses the acetification process to reach the required acetic degree and aging in wood. Phenolic compounds proved to be valid markers to differentiate vinegars according to their origin and elaboration process. Multivariate analysis of phenolic composition data including Linear Discriminant Analysis (LDA) and Artificial Neural Networks trained by Backpropagation (BPANN) classifies correctly vinegar according to the acetification process (LDA=92.5, BPANN =99.6) and origin (LDA=88, BPANN= 96.5). Good recalling classification rates were also obtained for aging periods. Indeed, phenolic aldehydes as syringaldehyde, vanillin, coniferaldehyde increase their concentration during aging. New trends in Sherry vinegars elaboration intends to shorten the production time. This is achieved by obtaining the acetic degree with submerged culture or by accelerating aging with chips. This communication presents the impact of both strategies on phenolic profile. Nowadays certain innovations to produce high quality vinegars include the type of wood used. Compounds released from it as (+)-taxifolin in the case of cherry wood or (+)-dihydrorobinetin for acacia wood are suitable chemical markers to characterize vinegars aged in these woods.

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Introduction

FAO/WHO (*1*) defines vinegar as any liquid fit for human consumption, produced exclusively from suitable agricultural products containing starch or sugars by a double fermentation process. The first step is the transformation of a fermentable carbohydrate source into ethanol by yeasts; the second step is the use of ethanol by acetic acid bacteria to produce acetic acid.

Vinegars´ quality is determined by the raw materials used and the acetification process involved in their elaboration. Raw materials used for vinegar making are very different: wine, cereals, rice, apples, cider are the most common but whey and honey can also be used to make vinegars.

Acetic acid bacteria (AAB) require oxygen to ferment alcohol. The availability of oxygen determines the growth of the AAB. If oxygen is not supplied, the bacteria grow just on the surface and the process is slow. Traditional vinegars are obtained by this surface culture method which involves large periods of time to obtain the acetic degree and meanwhile an aging process occurs simultaneously. Normally, these vinegars are produced in barrels where the contact with wood enhances the products organoleptic properties but a reduced production volume is obtained.

On the other hand, oxygen can be supplied into the liquid to be acetified by different systems (agitation, diffusions…) increasing its availability in a large volume of liquid. This fact allows the growth of a submerged culture AAB and a quick acetification process. By this method a volume of 30000 litres can be fermented in a short period of time as 36 hours. The installations need less space and the overall process is less expensive. Most wine vinegars consumed nowadays all over the world are produced in this way. However, volatile compounds are lost mainly due to the strong aeration in the system and sensorial properties are less appreciated than those of traditional vinegars.

Jerez Vinegars, Sherry Vinegars

Jerez vinegars are one of the most famous in the world. The production is around 4 millions of litres per year. Almost 200.000 litres are exported to the USA. In order to protect these vinegars, an official label of the Denomination of Origin Council is mandatory after bottling to ensure its origin. There are a few DO for vinegars in the world; Jerez vinegar, Condado de Huelva and Montilla Moriles in Spain and Aceto Balsamico Tradizionale di Modena and Aceto Balsamico Tradizionale di Reggio Emilia in Italy.

The conditions required to be included in the Jerez vinegars DO are directly linked to Sherry wine (*2*). Indeed, the same Council protects both Sherry wine and vinegar. The raw material must be wine from the white grapes Palomino, Pedro Ximenez or Muscat; the producing area is restricted to a geographical area in the Southwest of Spain; the aging process in oak woods by a period of time no less than 6 months restricted to Jerez, Sanlúcar and El Puerto area. Nowadays this area includes up to 48 wineries.

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Traditionally, Jerez vinegar is elaborated by surface culture; however, it is accepted to obtain the required acetic degree by a quick acetification process and submit the obtained vinegars to aging in barrels afterwards. The aging process defines the type of Jerez vinegar as follows: 6 months (Jerez vinegar), 2 years (Vinagre de Jerez Reserva) and Vinagre de Jerez Gran Reserva (10 years). Almost the 70% of the production is aged 6 months and a 30% aged for 2 years, being the most aged production very limited. The regulation fixes a minimum acidity of 70g/L (7ºAcetic acid) and 1.3g/L dry extract per degree of acetic acidity.

The analyses of chemical composition of Jerez vinegars demonstrate their special characteristics. Criteria to determine authenticity and traceability of the products are established on the basis of these scientific data. This paper presents the state of the art in the characterization of Jerez vinegars based on phenolic compounds as well as a discussion of recent advances in this field.

Authentication of Vinegars

The price of common vinegars is based on acetic degree. Thus, the earliest falsification was to sell synthetic acetic acid dilution as natural vinegar obtained by a natural fermentation process. ^{13}C isotope ratio mass spectrometry allows differentiating acetic acid obtained from fermentation from organic origin. However, adulteration is performed by mixing dilutions of natural vinegar and synthetic acetic acid. Recent advances to detect these additions are based on SNIF-RMN (Site Specific Natural Isotopic Fractionation Nuclear Magnetic Resonance Spectrometry) (*3*), an addition of just a 5% of synthetic acid can be detected. Below this limit, the practice becomes a non profitable trade. Moreover, isotopic methods allow to trace back the origin of chemically identical molecules. The $^{18}C/^{16}C$ ratio of water permits to differentiate vinegars from wine than those of raisins (*4*).

Vinegars produced from different raw materials can be differentiated by their content in polyalcohols as they are related to the substrate and persist after both alcoholic and acetic fermentations. Indeed, apple vinegar presents a particularly high content in sorbitol (3296 mg/L) and honey vinegar in manitol (958 mg/L) whilst vinegar obtained from alcohol lacks polyalcohol (*5*). However, polyalcohol content is not useful to characterize wine vinegars.

L-proline is a grape characteristic aminoacid and therefore it is present in their derivate products. Wine vinegars can present values up to 355-2187mg/L in Sherry vinegars (*6*), values 149-360 mg/L were reported in wine and lesser concentrations are suspicious of adulteration.

The efforts in the characterization of vinegars have been addressed to most expensive products: Jerez Vinegar and Aceto Balsamico Traditionale. Different aspects of their chemical composition specially related to aroma compounds have been revised recently (*7*, *8*). The present paper will focus on phenolic compounds as markers of quality vinegars.

Phenolic compounds are widespread in the plant kingdom and have been proposed as taxonomic marker in wines and different foods (*9–12*).

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First papers in the field described phenolic profile of Jerez vinegars by means of liquid chromatography coupled to diode array detectors (*13*). Suitability of this technique has been previously proved in many foods. Recently, these values have been compiled in the Phenol Explorer, a database on food polyphenols (*14*). Briefly, Jerez vinegars contain phenolic acids (gallic, protocatechuic, *p*-hydroxybenzoic, vanillic, caffeic, p-coumaric and ferulic acids and the tartaric esters of the hydroxycinnamic acids), aldehydes (vanillin, protocatechualdehyde, syringaldehyde, coniferaldehyde, p-hydroxybenzaldehyde), the flavanols (+)-catechin, (-)-epicatechin and caffeic ethyl ester and coumaric ethyl ester.

Multivariate statistical analyses proved useful to differentiate vinegars using their phenolic profile. A total of 92 wine vinegars representing most of the brands in the market at that moment were sampled and vinegars from two geographical close regions (Condado de Huelva and Montilla Moriles) were included. Linear discriminant analyses and also artificial neural network trained by backpropagation permits to differentiate vinegars obtained by surface culture from those obtained from submerged culture with following recalling rates: LDA (mean=92.5) and BPANN (mean =99.6) (*15*). Many variables in our data matrix did not fit normality requirements to be included in a linear model. The cause was that not detected compound means a zero. When several zeros are present in the data matrix it causes a lack of normality in the variable limiting its inclusion in further statistical analysis. However, the presence of a certain compound in a group of vinegar and its absence in another group must be a tool for differentiating these vinegars and should be taken into account for discriminating purposes. The solution to this problem was the use of artificial neural networks which do not require a normal distribution of data.

A short description of the statistical analysis of data is as follows: Phenolic compounds were used as variables to build up the data matrix. Cluster analysis explored data trends and natural groupings of samples. Clusters obtained by Wards' method showed some data trends including most vinegars obtained by submerged culture or those samples from Condado de Huelva. Principal Components Analysis revealed which variables contribute most to the variance of data; therefore, it was advisable to include them in the discriminate functions. Indeed, the first PC corresponded to those phenolic compounds presenting a hydroxycinnamic acid structure (caffeic, caffeoyltartaric, coumaric acid…) and the second PC to flavonoid structure compounds. Then, supervised pattern recognition was applied. Samples were divided into two sets (training set (75% of samples) and test set (25% of samples)). To validate both the goodness of the classification and the goodness of the prediction both training and prediction sets were repeated 10 times for different constitutions. This procedure was applied to differentiate between vinegars from very close geographical origins: Jerez, Condado and Montilla. Indeed, they are no more than 200 Km one from another. The recalling rates were 88.8 for LDA, and 96.5 for BPANN (*15*). Table 1 displays classification functions obtained.

On the other hand, aging in oak wood is an important aspect to develop organoleptic properties thus influencing quality. Indeed a Jerez category is determined by the aging period (less or more than 2 years). In order to determine whether or not phenolic profile could be useful to discriminate aged vinegars from

not aged vinegars, vinegar were sampled from wineries and divided according to their period of aging: less or more than 2 years as declared by producers. Some trends were observed in the concentrations of phenolic compounds however results obtained from BPANN do not allow to extract further conclusions (just a 90.4 for classification test and a 60% for the test set) (*16*). Nevertheless if aging period is strictly controlled in the samples used to construct the classification test, the assessed phenolic profile is useful to differentiate among vinegars with the following reference periods of aging (90, 180 and 225 days) as proved in a controlled experience. After 90 days of aging in oak wood barrels, the concentration in 5-(hydroxymethyl)-2-furaldehyde, 2-furaldehyde, vanillin, syringaldehyde and coniferaldehyde increased significantly ($p<0.05$). These compounds were used as variables to construct a function which classified correctly 100% of commercial samples from wineries (*17*).

The use of oak chips to shorten aging periods has been proposed as accelerated aging of vinegars. This accelerated process involves a prior acetification step to obtain the mandatory acetic degree. As ethanol is quickly transformed to acetic acid, another advantage is that producers also save the taxes corresponding to alcohol. Phenolic compounds did not change significantly ($p<0.05$) during the acetification of Palomino wine (*18*). After 15 days of macerating vinegar with chips, the extraction of phenolic compounds aromatic aldehydes, whisky lactones reached outstanding values (*19*). However, sensorial differences could be perceived by a trained panel between vinegar obtained by traditional Solera and criadera system and those obtained by infusion with oak chips (*20*) as vinegar aroma is complex and the contribution of esters formed during aging which contributes to overall perception.

In order to evaluate the sensorial impact of chemical composition on sensorial properties, a tasting procedure was validated (*21*) and aroma thresholds established in acetic acid solutions which are rather high than those obtained in watery solutions (Table 2). Certain substances released from wood were determined in vinegars to determine if their concentration were enough to be perceived by senses. Indeed, their concentration in vinegars obtained by accelerated aging with chips after 15 days are higher than the threshold limits. Furfural (2-furancarboxyaldehyde) originated from the degradation of monosaccharides produced by the partial hydrolysis of hemicellulose contributes to the dried fruit characteristic, particularly toasted almonds values up to 10.7 mg/L after 90 days of aging with chips have been reported whilst its limit is 6.2 mg/L in acetic acid solutions of 6%. Eugenol (2-methoxy-4-(2-propenyl)-phenol), a volatile phenol is produced by the breakdown of lignin during wood toasting and gives an aroma related to flavours of spices, cloves and smoke. Vanillin (4-hydroxy-3-methoxybenzaldehyde) a by product of lignin degradation during toasting is related to the vanillin descriptor. Indeed the threshold was determined at 94.4 µg/L in acetic acid solutions and the concentration in Jerez vinegars aged in barrel is around 2 mg/L and in vinegar aged with chips up to 14.7 mg/L.

Table 1. Coefficients for the variables in the classification functions to discriminate vinegars. Variables are the concentrations of the phenolic compounds

Functions	*T*	*GA*	*CT*	*HMF*	*CM*	*GE*	*CA*	*COEF.*
Z_{JZ}	0.01928	0.08229	0.18928	0.10440	-0.60414	-0.02380	0.79207	-5.03973
Z_{CH}	0.14120	0.06482	0.05127	0.04562	0.22997	0.03870	-0.05668	-6.57400
Z_{MM}	0.02434	0.19139	0.16152	0.01955	-1.89564	0.37827	-0.05668	-9.99663
Z_{XX}	0.00286	0.03115	0.09779	0.01244	-0.35961	-0.02810	-0.01548	-2.34032
Z_{JZR}	-0.0053	0.1963	0.3724	0.0321	-2.0264	-0.3841	0.7139	-10.9052
Z_{MMR}	0.04032	0.01895	0.03961	0.00862	-0.12258	0.00483	0.09397	-3.54368

T: tyrosol; GA: gallic acid; CT: caftaric acid; HMF: 5-(hydroxymethyl)-2-furaldehyde; CM: *p*-coumaric acid; GE: gallic ethyl ester; CA: cafeic acid; COEF.: coefficient; JZ: Jerez; CH: Condado de Huelva; MM: Montilla-Moriles; XX: commercial submerged culture; JZR: Jerez submerged culture; MMR: Montilla-Moriles submerged culture.

In summary, the state of the art concerning the role of phenolic compounds in Sherry vinegars encompasses the description of vinegar composition by liquid chromatography coupled with DAD, the statistical analysis by multivariate methods which permits to differentiate vinegars by their origin and aging period. Special contributors are those phenolic aldehydes released from lignin degradation from the oak barrels where vinegar is aged. Still a challenging issue to investigate is the search of those molecules formed during the elaboration process or released from wood with a high impact on sensorial properties of the product.

Table 2. Threshold Log Standard Deviation and observed Range for 5 aroma compounds added to Milli-Q water and 6% v/v acetic acid solution

Samples	*Group Threshold*	*Log Standard deviation*	*Observed range of thresholds*	*Number of tasters*
1. Acetaldehyde in Milli-Q water	80.2 μg/L	0.086	50 -270 μg/L	7
2. Acetaldehyde in 6% acetic acid solution	401.8 μg/L	0.086	250-1350 μg/L	7
3. Benzaldehyde in Milli-Q water	104.2 μg/L	0.241	25-200 μg/L	8
4. Benzaldehyde in 6% acetic acid solution	157.8 μg/L	0.090	100-280 μg/L	8
5. Ethyl acetate in Milli-Q water	22.3 mg/L	0.100	10-75 mg/L	8
6. Ethyl acetate in 6% acetic acid solution	90.8 mg/L	0.065	50-200 mg/L	8
7. 2-Furaldehyde in Milli-Q water	208 μg/L	0.222	100-2000 μg/L	8
8. 2-Furaldehyde in 6% acetic acid solution	6.2 mg/L	0.235	5-15 mg/L	8
9. Vanillin in Milli-Q water	32.2 μg/L	0.056	10-60 μg/L	8
10. Vanillin in 6% acetic acid solution	94.4 μg/L	0.300	30-960 μg/L	8

Foresight for High Quality Vinegars Characterization

Vinegars industry faces the challenge of producing high quality products with new organoleptic properties and obviously a profitable elaboration from an economic point of view. Nowadays, Protections of Designations of Origin for vinegars are devoted to white vinegar; the production of red vinegars is smaller and represents a new product development opportunity.

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On the other hand, wood contact determines sensorial properties and the role of different woods on vinegars quality needs evaluation. Indeed, most papers in the field are devoted to aging in oak. Oak is a suitable wood for wine making as it limits oxygen transfer through its pore avoiding wine oxidation. However, vinegar elaboration requires a large pore to promote oxygen transfer for the growth of the acetic acid bacteria. The WINEGAR project (EU funded, COOP-CT-2005-017269) aimed to establish the role of different woods (oak, chestnut, cherry and acacia) on traditionally made red wine vinegars.

This work presents recent results of this project. The purpose is to ascertain which phenolic compounds are relevant for authenticity purposes as an advance in future characterization of products produced with these woods.

The experimental work was accomplished with non toasted barrels as the purpose was improve vinegar making process in traditional methods by having a good oxygen transfer. Acetification was a surface culture and red wine substrate from Grenache variety.

It was noticeable the absence of aromatic aldehydes from lignin degradation in vinegars aged in oak wood opposite to our previous findings. The cause was the selection of non toasted wood.

(+)-Taxifolin can be pinpointed as cherry wood marker. Indeed, vinegars in contact with cherry barrels present this flavonoid in increasing concentration up to 3.09 ± 0.06 after a year of aging in wood (Figure 1). Conversely, (+)-taxifolin decreased its concentration after a year of storage in bottle. This fact has to be taken into account to trace back aged products or products in the shell of the markets for a long time. *Prunus avium* or cherry tree wood differs from oak wood in the large amount of flavonoids compounds, namely absent in oak wood (*22*).

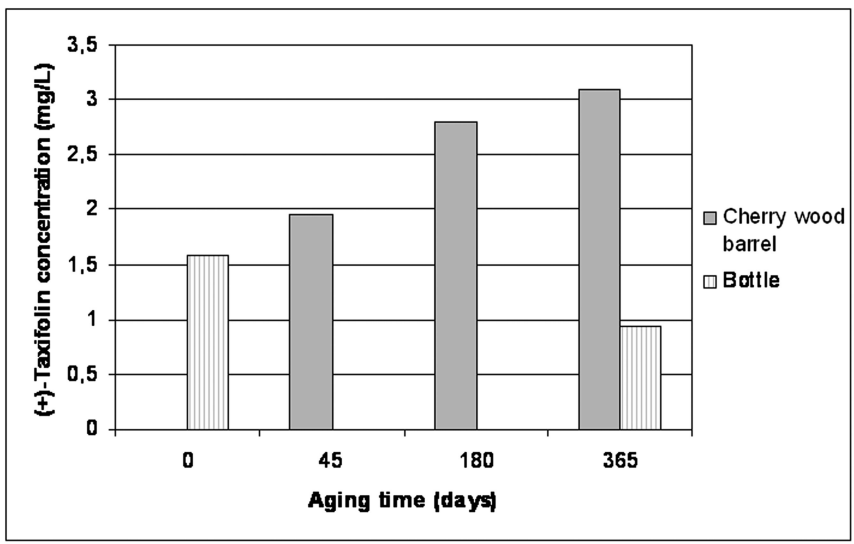

Figure 1. (+)-Taxifolin concentration during aging in cherry wood barrel and bottle.

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Vinegars elaborated in contact with chestnut wood present a remarkable high amount of gallic acid due to the high amount of hydrolysable tannins in this wood (Figure 2). During acetification in traditional methods, ethanol is transformed slowly in acetic acid and it is normally present even in final products up to 2º v/v. The formation of ethyl gallic ester is favoured and it was determined.

Main descriptor of acacia (*Robinia pseudoacacia*) wood is (+)-dihydrorobinetin which was isolated from vinegars aged in acacia barrels and undoubtly identified by ^{13}C-NMR (Figure 3). Indeed, its concentration increases during the acetification and values may reach up to 438.58 ± 2.16 after a year of contact in wood. This dihydroflavonol has a remarkable in vitro antioxidant activity increasing the overall AA value of vinegar in a 8.6-13.65%.

The contribution of the kind of wood was explored assessing the phenolic composition of vinegars elaborated in the four woods under study keeping the rest of variables as AAB inoculum wine substrate constant. The experiment revealed that phenolic profile was useful to construct discrimination functions to correctly classify the samples with a 98.6% recalling rate. Additionally, a sensory panel could differentiate by triangle tests vinegars produced in each kind of wood (Table 3). The descriptive analysis shows higher scores for fruit descriptor for vinegar elaborated in cherry wood whilst vinegar elaborated in oak wood presents a remarkable high vanilla score (*24*).

The role of the acetification process on phenolic composition is another important feature to be determined. If white wine vinegars are obtained by submerged culture, no significant differences in phenolic acids could be observed (*18*). Even if the acetification is by surface culture and dissolved oxygen is rather limited both (+)-catechin and resveratrol are oxidized (*24*).

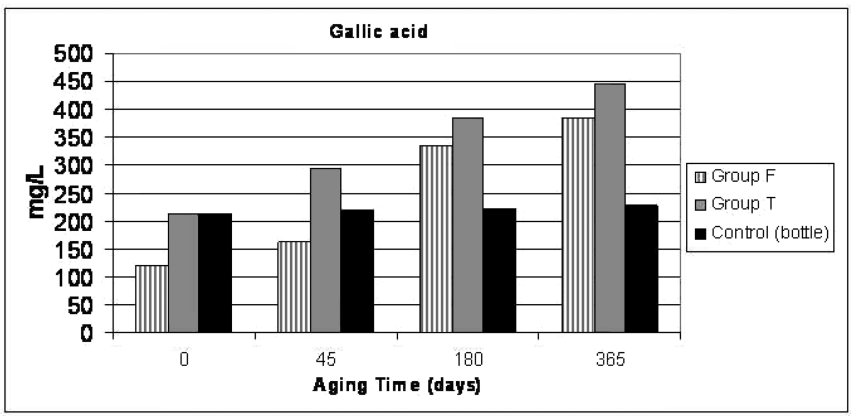

Figure 2. Gallic acid concentration of vinegars aging in chestnut wood barrels (Group F and T) and bottle (control).

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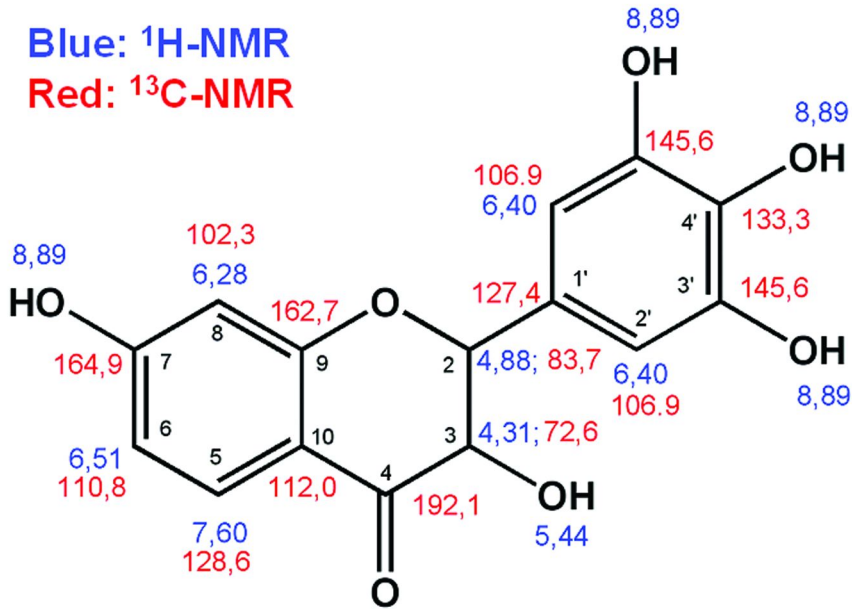

Figure 3. [1]H and [13]C NMR chemical shifts data of isolated dihydrorobinetin from vinegar.

Recent efforts have been paid to anthocyanin compounds and their changes after the acetification with submerged culture. The identification of a total of 17 anthocyanin derived pigments (pyranoanthocyanins and ethyl-linked compounds) in red wine vinegars is achieved for the first time (*25*). The effect of the submerged acetification process on phenolic composition is as follows: vitisin-type and ethyl-linked compounds increase their concentrations, while the concentration of monomeric anthocyanins, phenolic acids (ferulic acid, caffeic acid and caftaric acid) and flavan-3-ol ((+)-catechin) decreases probably due to condensation the aforementioned condensation reactions. The concentration of (+)-catechin also decreases during acetification with surface culture (*24*).

Thermic treatment of the acacia wood is crucial for (+)-dihydrorobinetin concentration (Figure 4) as toasting can decrease its concentration in vinegar from 10 to 20 times (*23*).

Production of red wine vinegars is likely to increase and become more diverse. As current knowledge is restricted to this experience with Cabernet Sauvignon wine, further research will be needed. Studies in the field will address the selection of suitable substrate to obtain red wine vinegar with attractive and stable colours.

On the other hand, condiments made from Muscat grape or different fruits are being developed and authenticity criteria will have to be addressed towards those new products. Indeed, these condiments are actually being developed in the Asian countries for their sensorial and healthy properties. Indeed, vinegar increases the salty perception of foods and represents an alternative when salt consumption must be restricted in case of hypertension. Further characterization of their chemical composition is required to relate them with possible healthy properties of vinegars.

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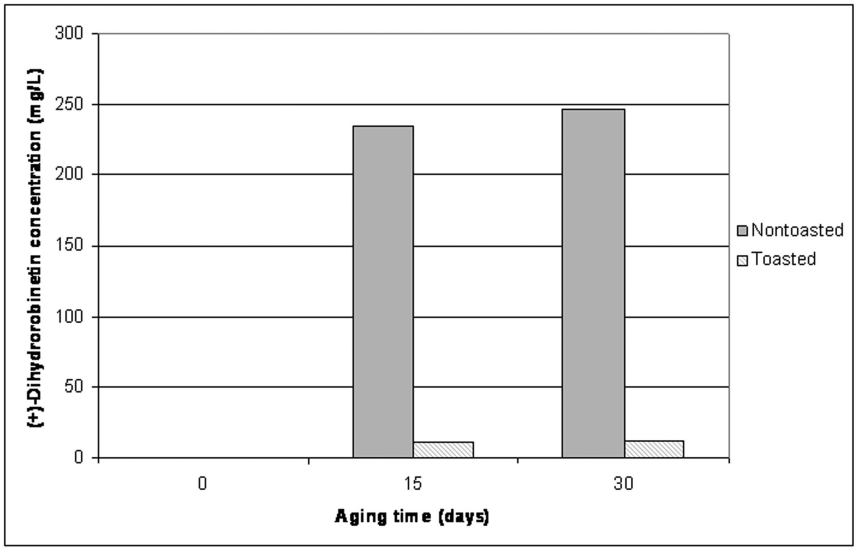

Figure 4. (+)-Dihydrorobinetin concentration (mg/L) in vinegar samples aged with different thermal treatment of acacia wood chips for different aging times.

Table 3. Results of triangle test of vinegar aged in different wood barrels and aging period (significant level for sensory differencces)

Aging period		*Acacia*	*Cherry*	*Chestnut*	*Oak*
45 days	Acacia	–	n.s.	5%	1%
	Cherry		–	1%	5%
	Chestnut			–	5%
	Oak				–
180 days	Acacia	–	n.s.	5%	0.1%
	Cherry		–	0.1%	5%
	Chestnut			–	0.1%
	Oak				–
365 days	Acacia	–	n.s.	1%	0.1%
	Cherry		–	1%	1%
	Chestnut			–	0.1%
	Oak				–

n.s.: non-significant

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Chapter 15

Proteolytic Peptides as Molecular Markers of Species' Authenticity in Cheeses

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Different mammalian species usually show differences in the casein sequences. Due to the proteolytic process taking place during cheese production and ageing, caseins from different species can generate homologous but not identical peptides. These peptides can be used as molecular markers in order to determine the animal species from which the milk is provided. In the present work LC/ESI-MS methodologies have been applied for detecting species' specific oligopeptides in different cheeses. Fragment 1-23 of α_{S1}-casein has been used as marker for the use of cow's milk as ingredient in two different types of sheep cheeses ("Pecorino"). In both cheeses the presence of cow-derived caseins was confirmed, albeit in one of them only sheep milk was declared among the ingredients. Fragment 16-25 of β-casein was used in order to confirm the actual use of the declared water buffalo's milk as ingredient in mixed cow-water buffalo "Grana" cheeses.

Cow's milk is the main ingredient for cheese production all over the world, though many cheese products are made starting from milk of different mammalian species, such as sheep, goat, water buffalo, and others.

The production of cheeses derived from sheep milk is very common in European and Asian countries, with Greece, France, Spain and Italy as world leaders. Also water buffalo's milk is used for the production of several cheeses, among them the most famous is probably the Italian "mozzarella". Cheeses made

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from non-cow's milk are often perceived by the consumers as products with superior quality attributes, and this perception is usually enforced by the higher market prices. High prices are also justified by the lower availability and by the seasonal fluctuations of milks coming from mammalian species other than cows. Many cheeses produced by non-cow's milks also bear a Protected Designation of Origin (PDO) which explicitly forbids the use of cow's milk for their preparation. In this context, the presence of undeclared (and cheaper) cow's milk as ingredient of cheeses supposedly made from milk of other species is a common fraud (*1*).

There are several methods based on protein determination reported in the literature for detecting sheep and goat cheese adulteration with cow's milk (*2–4*). The European official method is based on different mobility of gamma casein obtained after plasmin hydrolysis (*5*), but many other methods have been proposed for identifying adulteration of sheep cheeses with cow's milk: capillary electrophoresis of whey proteins (*6*, *7*), liquid chromatography of denatured caseins (*8*) and immunological methods (*9–11*). However, the application of these methods is often hampered by protein breakdown due to proteolytic enzymes (*12*). PCR-based methods aimed at identifying cow's DNA have also been proposed (*13*).

Recently, we proposed the use of the proteolytic peptides, generated from the casein breakdown which takes place during the production and the ageing of cheeses, as markers for the mammalian species from which the milk is produced. In particular, LC/ESI-MS analysis of homologous, but not identical, proteolytic peptides derived from α_{S1}-casein allowed the rapid and reliable assessment of the presence of cow's milk in cheeses supposedly made only from sheep milk (*14*). In a similar approach, LC/ESI-MS/MS analysis of the peptides generated by plasmin digestion of caseins extracted from cheeses allowed the detection of the presence of cow's milk in sheep cheeses or of sheep's milk in goat cheeses (*15*).

In the present paper we present the analysis of the oligopeptide fraction of different types of cheeses in order to identify the species providing the milk used for cheese production, discussing the potentialities and the limitations of this approach for cheese authentication.

LC/ESI-MS Method for the Analysis of the Peptide Fraction in Cheeses

The method for extracting, concentrating and analyzing the oligopeptide fraction of cheeses is essentially that previously reported by our group, with few modifications (*14*). Briefly, grated cheese (10 g) was placed in a 250 mL beaker. Forty-five mL of 0.1M HCl was then added, followed by homogenization for 2 min. The homogenates were then centrifuged for 30 min at 4°C and filtered through a Buchner funnel. The resulting solution was extracted three times with ethyl ether. The aqueous solution was filtered with HVLP filters with a cut-off of 0.45 μm. The filtrate was then evaporated to dryness, redissolved in a 0.1% solution of formic acid in water and then ultrafiltered with filters with a nominal cutoff of 10 kDa. Three mL of the filtrate was evaporated to dryness, redissolved in 0.5 mL of a 0.1% solution of formic acid in water and analyzed by a UPLC

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(Waters Acquity) interfaced with a single quadrupole mass spectrometer (Waters SQD). Chromatographic column: C_{18} Acquity UPLC BEH300, 1.7 µm, 2.1x150 mm; volume injected 2 µL; flow: 0.2 mL/min; column temp. 35°C; gradient: 7 min isocratic 100% eluent A (water + 0.2% acetonitrile + 0.1% formic acid), from 7 to 50 min from 100% A to 50% A / 50% B (acetonitrile + 0.1% formic acid), plus washing and reconditioning.; ESI-MS parameters: positive ion mode, capillary voltage 3.2 kV, cone voltage 30 V, T source 150°C, T desolvation 200°C, cone gas (N_2) 100 L/h, desolvation gas (N_2) 650 L/h, full scan acquisition from 100 to 2000 m/z, scan time 1 sec., interscan delay 0.1 sec. "In-source" generated fragments have been used for peptide identification.

Determination of the α_{S1}CNf(1-23) Peptide for Assessing the Presence of Cow's Milk in Sheep Cheeses

The oligopeptide fractions extracted from two "Pecorino" cheeses available on the Italian market were first analyzed in order to assess the presence of cow-derived peptides. In particular, according to our previous work (*14*), we decided to use as marker the fragment 1-23 of the α_{S1} casein (α_{S1}CNf(1-23)). This peptide is mainly produced by chimosin during the first steps of the cheese production process, but usually can also be detected also after long ageing times or in highly proteolyzed cheeses. The sequences of cow and sheep α_{S1}CNf(1-23) bear few differences, as reported in Table I.

Table I. Differences of the α_{S1}CNf(1-23) peptide in cows and sheep. The most common isoforms are reported. The differences are marked in bold.

Origin	*α_{S1}CNf(1-23) sequence*	*Average MW*
Cow	RPKHPIKHQGL**PQ**EVLNENLLRF	2764 Da
Sheep	RPKHPIKHQGL**SP**EVLNENLLRF	2723 Da

The sequences are very similar, which is an important feature for their use as molecular markers, since a similar behavior towards the proteolytic enzymes is expected in the cheese matrix. Nevertheless, they are different enough to be discriminated by mass spectrometric techniques.

The first sample analyzed was a "Pecorino Toscano", a PDO (Protected Designation of Origin) cheese. The Disciplinary of Production for this cheese only allows the use of pure sheep milk and, accordingly, on the label only sheep milk was declared.

A full scan chromatogram of the peptide fraction is reported in Figure 1. As can be seen, the peptide fraction is quite rich, which is indicative of a product which did not undergo a very long ageing time: in fact this sample of "Pecorino Toscano" had only about 30 days of aging.

From the Full Scan Chromatogram, Extract Ion Chromatograms can be obtained by selecting and extracting ions of interest. The ions having m/z 908.7, 681.8 and 545.5, corresponding respectively to the multiprotonated and

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multicharged molecular forms $(M+3H^+)^{3+}$, $(M+4H^+)^{4+}$ and $(M+5H^+)^{5+}$ of ovine α_{S1}CNf(1-23), were extracted. Then, the same procedure was repeated extracting the ions having m/z 922.5, 692.0 and 553.8, corresponding respectively to the multiprotonated and multicharged molecular forms $(M+3H^+)^{3+}$, $(M+4H^+)^{4+}$ and $(M+5H^+)^{5+}$ of bovine α_{S1}CNf(1-23). The two extracted chromatograms are reported in Figure 2.

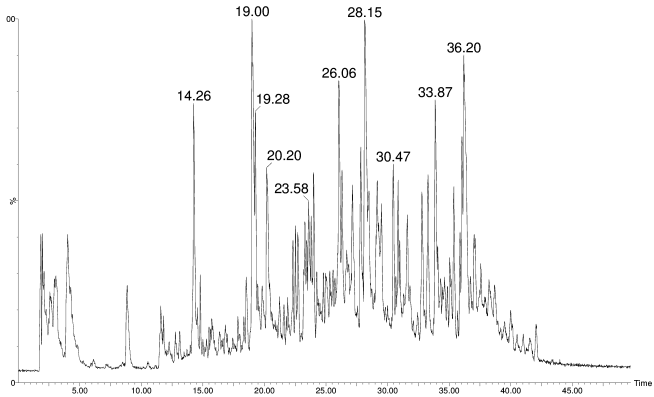

Figure 1. Full Scan chromatogram of the peptide fraction extracted from a "Pecorino Toscano" cheese.

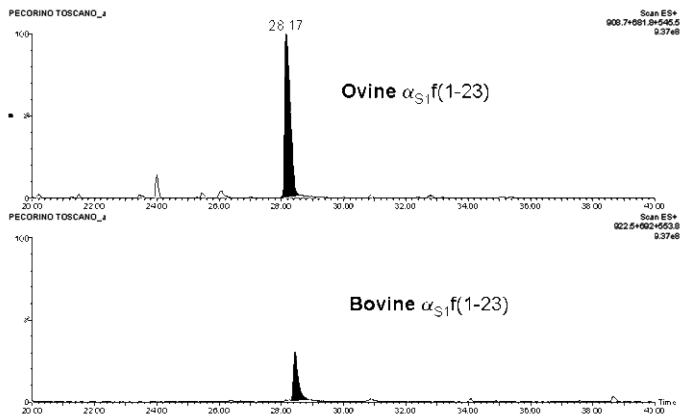

Figure 2. Extract Ion chromatograms of the peptide fraction of "Pecorino Toscano" cheese obtained by monitoring the ions corresponding to ovine (top) and bovine (bottom) α_{S1}CNf(1-23).

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In order to confirm the peak identity, the MS spectra corresponding to the two compounds were also analyzed. The mass spectrum of ovine α_{S1}CNf(1-23) (Figure 3) showed, together with the multicharged molecular ions, several diagnostic fragments (indicated with standard peptide fragment notation), that allowed the confirmation of its identity. The mass spectrum of the putative bovine α_{S1}CNf(1-23) is reported in Figure 4. Again, besides the multicharged molecular ions, several diagnostic fragments are present (indicated with standard peptide fragment notation), that are totally consistent with the proposed identification.

Even the relative retention time of the second peak to the first one (relative Rt = 1.01) was found to be totally consistent with that previously found for the two α_{S1}CNf(1-23) peptides (*14*).

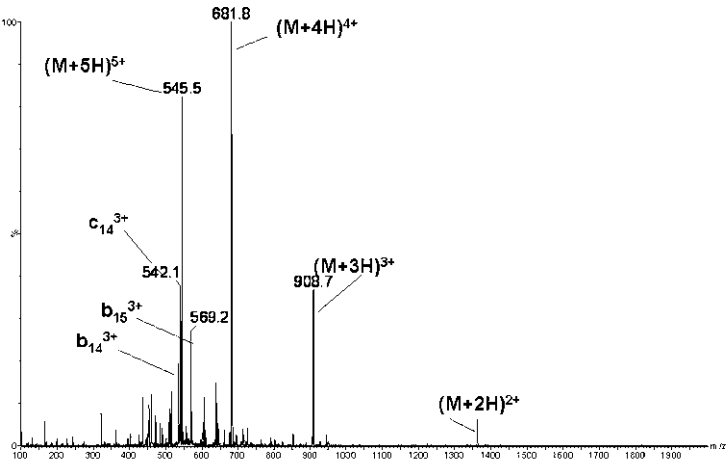

Figure 3. ESI mass spectrum corresponding to the peak at Rt=28.2 min. Multicharged molecular ions and fragments are consistent with its identification as ovine α_{S1}CNf(1-23).

Thus, the data indicate a significant presence of cow's milk in the milk used for producing this "Pecorino" cheese. The relative percentage for the two peptides, calculated by the corresponding areas of the chromatographic peaks (81% sheep peptide, 19% cow peptide), given the similarity of the two structures, is likely to be a good indicator of the relative amount of the two molecules. Albeit this might not directly reflect the actual percentage of cow's milk (mainly due to differences in casein content and reactivity towards proteolytic enzymes), in any case the presence of an undeclared ingredient can not be neglected.

Subsequently, also a "Pecorino Romano" cheese was also analyzed in this way. The Full Scan chromatogram is reported in Figure 5.

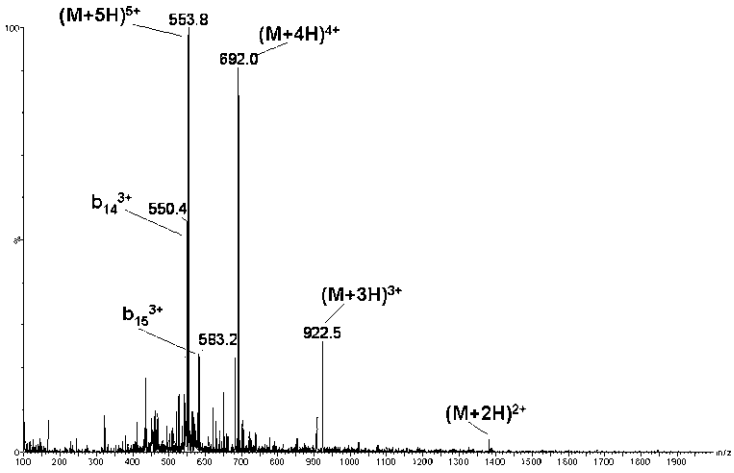

Figure 4. ESI mass spectrum corresponding to the peak at Rt=28.5 min. Multicharged molecular ions and fragments are consistent with its identification as bovine α_{S1}CNf(1-23).

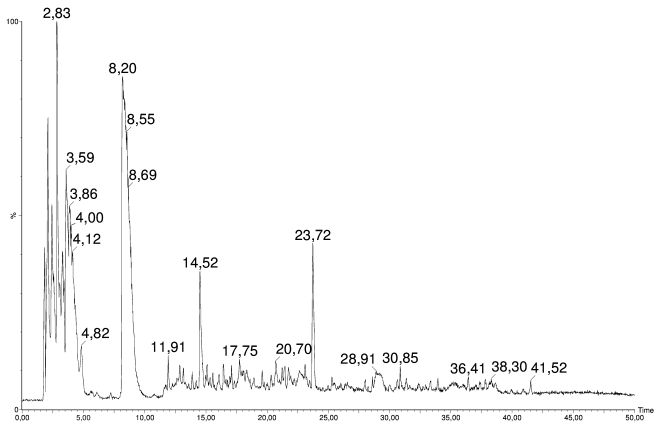

Figure 5. Full Scan chromatogram of the peptide fraction extracted from a "Pecorino Romano" cheese.

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The higher intensities of the first peaks (free amino acids) and the lower intensities of the peaks between 10 and 50 minutes (oligopeptides) is indicative of a highly proteolyzed cheese, and actually the "Pecorino Romano" cheeses are usually aged for several months. In this case the α_{S1}CNf(1-23) peptide is expected to be present in a quite low amount. The ions corresponding to the two peptides, ovine and bovine, were extracted as before. The results are given in Figure 6.

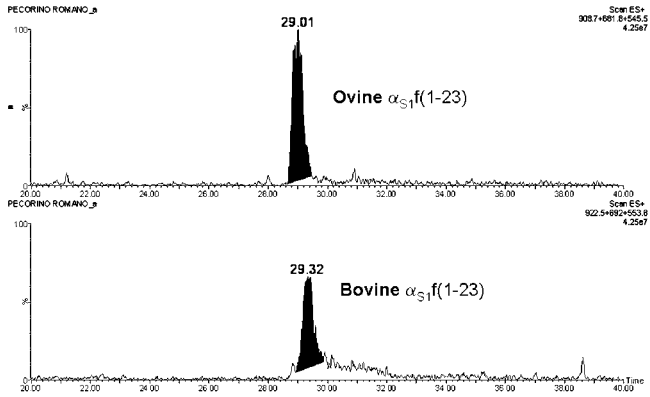

Figure 6. Extract Ion chromatograms of the peptide fraction of "Pecorino Romano" cheese obtained by monitoring the ions corresponding to ovine (top) and bovine (bottom) α_{S1}CNf(1-23).

Again, albeit present in much lesser amount, the identity of the second peak was confirmed by checking its mass spectrum, reported in Figure 7: the multicharged and fragmentation peaks confirm its identification as the bovine α_{S1}CNf(1-23), also consistent with the spectrum reported in Figure 4 for the "Pecorino Toscano" cheese. Its identity as bovine α_{S1}CNf(1-23) was also confirmed by the relative retention time (relative Rt = 1.01).

In this case, the product did not bear a Protected Designation of Origin, thus no Disciplinary of Production could be used as reference; moreover in the ingredient list the generic term "milk" (with no reference whatsoever to the species of origin) was used. The relative abundance of the two peptides, calculated by the corresponding areas of the chromatographic peaks (60% ovine, 40% bovine) testifies for a consistent use of cow's milk, but in this case its presence, albeit ethically questionable, was not explicitly excluded in the label.

It can be noted how the methodology proposed here allowed us to assess the identity of the peptide chosen as molecular marker even in a highly proteolyzed sample, containing small amount of α_{S1}CNf(1-23).

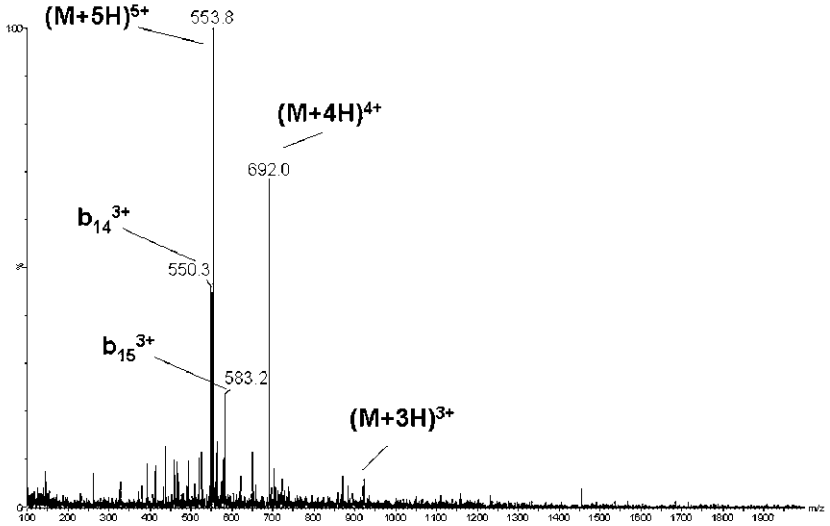

Figure 7. ESI mass spectrum corresponding to the peak at Rt=29.3 min. Mutlicharged molecular ions and fragments are consistent with its identification as bovine α_{S1}CNf(1-23).

Determination of the βCNf(16-25) Peptide for Assessing the Presence of Water Buffalo's Milk in Mixed "Grana" Cheeses

The third sample analyzed was a "Grana" cheese claimed to be made from water buffalo's milk. In the label, however, both cow's milk and water buffalo's milk were declared as ingredients, in this respective order, thus implying that cow's milk was more abundant than buffalo's milk.

The LC/MS chromatogram of the peptide fraction is reported in Figure 8.

Again, this chromatogram is indicative of a highly proteolyzed cheese, which is typical for aged "Grana" cheeses. The most intense signal in the peptide fraction corresponds to non-proteolytic pseudopeptide-like molecules, previously reported to be abundant in this type of cheese (*16*), which could not be used as species' markers. The determination of the α_{S1}CNf(1-23) peptide according to the methodology previously described in this case failed to detect any signal. However, the full scan acquisition allowed to thoroughly analyze the chromatogram in order to find other diagnostic peptides. A suitable marker peptide was finally identified, originating from a β-casein region: the fragment 16-25 of the β casein, also including three phosphate groups (βCNf(16-25)3P). This fragment has already been reported to be present in "Grana"-like hard cheeses, including Parmigiano-Reggiano and Grana Padano (*17*, *18*). The sequence of the homologous peptides in cows and water buffalos are reported in Table II.

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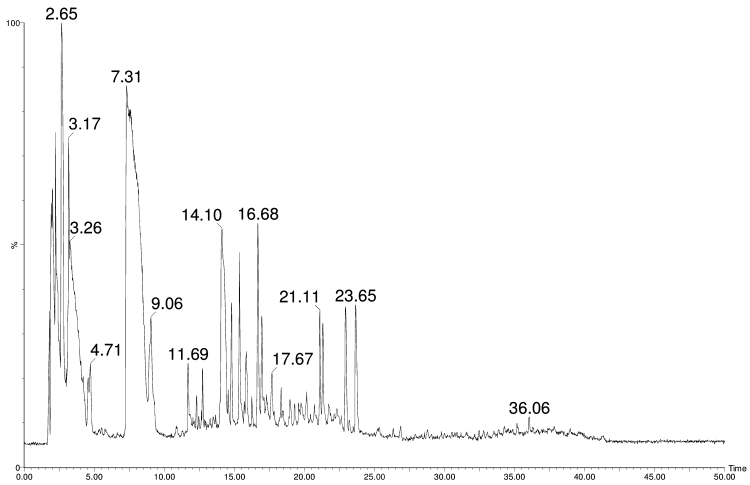

Figure 8. Full Scan chromatogram of the peptide fraction extracted from a "Grana" cheese partly made from water buffalo's milk.

Table II. Differences of the βCNf(16-25)3P peptide in cows and water buffalos. The most common isoforms are reported. The differences are marked in bold.

Origin	*βCNf(16-25)3P sequence*	*Average MW*
Cow	LpSpSpSEESIT**R**	1348 Da
Water Buffalo	LpSpSpSEESIT**H**	1329 Da

From the full scan chromatograms the ions corresponding to the doubly protonated (doubly charged) molecules were extracted (m/z 665.5 for the cow's peptide, m/z 675.0 for the water buffalo's peptide). The corresponding chromatograms are reported in Figure 9.

In order to confirm the peak identity, the MS spectra corresponding to the two compounds were also analyzed. The mass spectrum of the buffalo's βCNf(16-25)3P peptide (Figure 10) showed, together with the multicharged molecular ions, several diagnostic fragments (indicated with standard peptide fragment notation) that allowed the confirmation of its identity. The mass spectrum of the cow's (βCNf(16-25)3P peptide is reported in Figure 11. Again, besides the multicharged molecular ions, several diagnostic fragments (indicated with standard peptide fragment notation) were present that were totally consistent with the proposed identification.

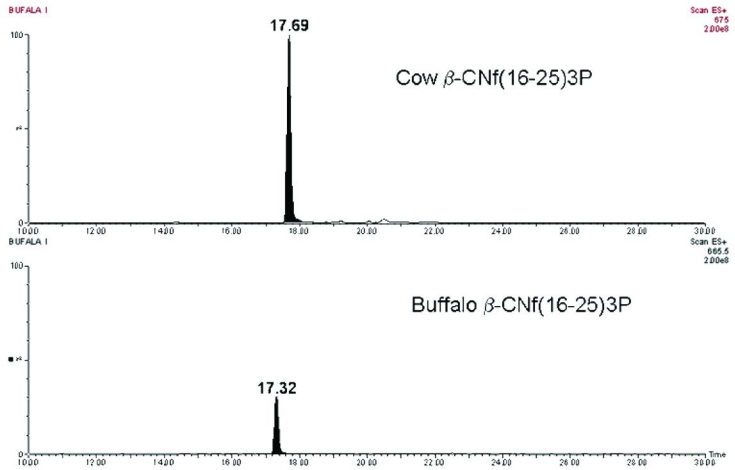

Figure 9. Extract Ion chromatograms of the peptide fraction of "Grana" cheese obtained by monitoring the ions corresponding to buffalo's (top) and cow's (bottom) βCNf(16-25)3P peptide.

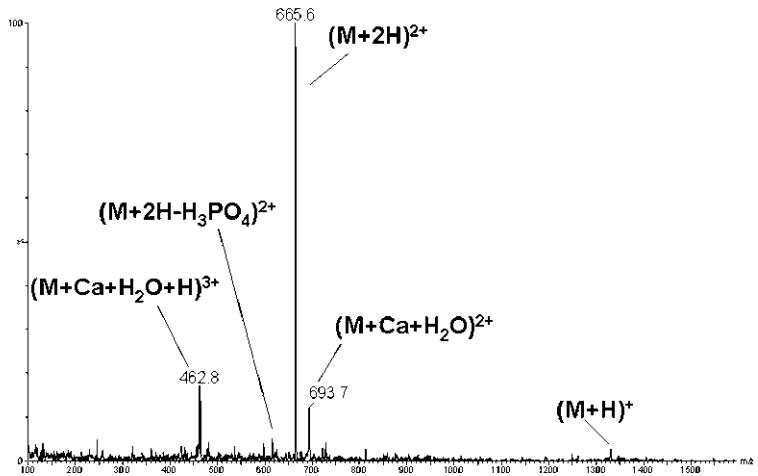

Figure 10. ESI mass spectrum corresponding to the peak at Rt=17.3 min. Multicharged molecular ions and fragments are consistent with its identification as water buffalo's βCNf(16-25)3P peptide.

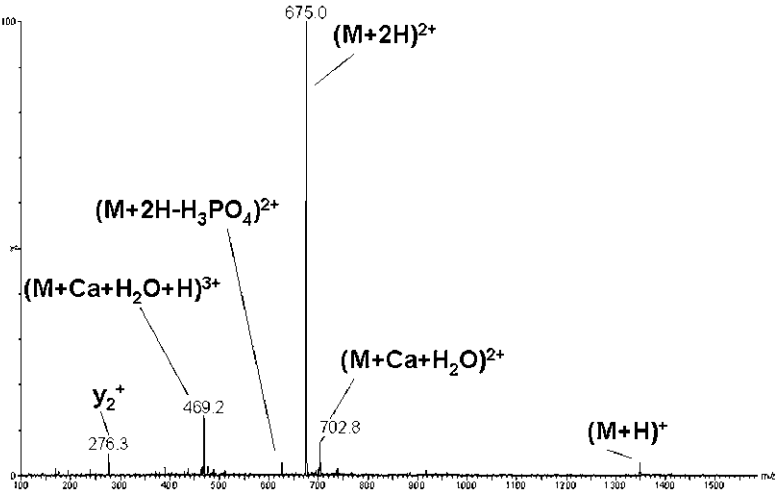

Figure 11. ESI mass spectrum corresponding to the peak at Rt=17.7 min. Mutlicharged molecular ions and fragments are consistent with its identification as cow's βCNf(16-25)3P peptide.

Thus, the presence of water buffalo's milk as ingredient of this "Grana" cheese has been confirmed, albeit with the simultaneous presence of cow's milk. Actually, the relative percentage of the two peptides, calculated by the corresponding areas of the chromatographic peaks (23% water buffalo's peptide, 77% cow's peptide), indicates a more relevant presence of cow's milk as ingredient, a fact correctly declared on the label.

Conclusions

The use of proteolytic peptides, naturally present in the oligopeptide fraction of cheeses, as molecular markers for species identification has been demonstrated to be a very useful tool for assessing cheese authenticity. The LC/ESI-MS method here presented is simple to perform and can potentially yield much information, due to the richness of the oligopeptide fraction in cheeses. According to the species to be identified, different peptides may be used for the purpose, making this method quite flexible. Even if in principle false negative results can not be excluded, in the case of lack of detection of a particular marker peptide due to its low amount in the fraction, this event can be minimized by the possibility of studying many different peptide sequences. On the other side, the discriminative power of mass spectrometry makes the possibility of false positive identifications practically nil, making this method an ideal tool for detecting fraud concerning cheese authenticity.

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Chapter 16

Authenticity of Caffeine Containing Beverages

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This paper deals with the authentication of tea and coffee and the beverages made from. Issues in the authentication of tea are the discrimination of the geographic origin and the differentiation of the type of tea (black, green, oolong, and white). For coffee, the differentiation of the varieties C. *arabica* and *robusta* is relevant, which can be achieved by the determination of 16-O-methylcafestol in both roasted and green coffee. Other possibilities are the area of growth which might be detected using the chlorogenic acid pattern in green coffee beans. Other approaches, such as isotope ratios, NIR spectroscopy, and determination of volatiles have been also employed. The authentication of instant coffee is possible using the determination of carbohydrates and the ratio of xylose to glucose. Other carbohydrates might additionally be used for that purpose.

Introduction

Tea and coffee are the most relevant caffeine containing beverages according to their consumption figures. Other beverages, such as guarana or the caffeinated softdrink are not covered in this chapter. The authentication of tea and tea products was already discussed in the 2005 symposium on the authentication of foods and wine (*1*). Therefore in this case only more recent developments and still unsolved problems are discussed. One trend in the field of tea products are special extracts having a completely different pattern of compounds, e.g. a tea extract with very high amounts of theanine and theogallin (*2*). White tea authentication is still kind of a problem as no international accepted definition for this type of teas exists.

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In case of coffee, the other relevant methylxanthin containing beverage, there are a few issues to be mentioned. The differentiation of C. *arabica* and *robusta* is an issue as well as the detection of the geographic origin of the samples. It is claimed that it is possible for a trained person to identify sensorically a Robusta or an Arabica sample, however, in a roast and ground sample this will not always work. Another sensory approach is tasting where trained people also are thought to be able to find out whether or not Robusta in relevant amounts is in the samples.

Authentication of Tea

For the discrimination of green and black tea an international data collection using validated standard methods has been completed and is ready to be published (*3*). Those standards have been used to determine the total phenolics content by a Folin-Ciocalteu assay and the catechins along with caffeine and gallic acid by an HPLC procedure (*4*, *5*). In the data collection all participating labs worldwide used this methodology. Moreover, the data collection was combined with ring test so non-complying labs could be excluded and their results are not in the database. The database contains more than 600 green and black teas. In most cases a clear discrimination between green and black teas is obtained. There are quite a few exceptions mostly caused by Darjeeling samples which have a quite high catechin contents. Moreover, the proportion of the catechins to total phenolic is more than 0.5 (50%) in case of nearly all Darjeeling samples. It is possible to determine also other compounds such as theogallin, gallocatechin, theobromine, and epimers such as gallocatechin gallate and catechin gallate, however, this was not specified in the original standard and consequently most participating laboratories did not do it (Engelhardt 2010, unpublished results).

White teas are a special issue as no generally accepted definition exists (*6*). Recently, the ISO working group on tea discussed a technical document which proposed a manufacture based definition of white tea (*7*). A differentiation of green and white tea by the catechin and total phenolic contents or ratios is not possible (*8*). Looking for a marker compound for white teas led to the identification of a myricetin triglycoside (Figure 1) which had not been identified before in tea (*9*). Later it has been shown that most of the green teas and also some of the black teas analyzed contained small amounts of this triglycoside. Also there is no significant difference between green and white tea in the content or the pattern of flavonol glycosides (*8*).

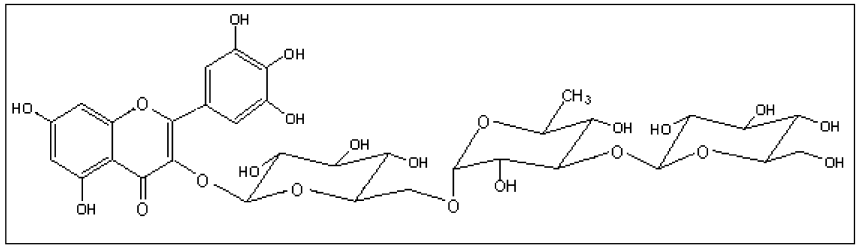

Figure 1. Myricetin-3-O-rhamnodiglucoside.

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If it holds true that a possible definition of white tea is based on manufacture it might be hypothesized that a determination of remaining enzyme activity might be useful as no enzyme deactivation seems to be involved in white tea processing (*10*).

Analysis of metal content was always used as a tool to identify the origin of tea. Often LDA was employed for a classification (*11*). Recently, a combination of isotope ratio (δN, δD) and trace elements (^{49}Ti, ^{53}Cr, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{71}Ga, ^{85}Rb, ^{88}Sr, ^{89}Y, ^{93}Nb, ^{111}Cd, ^{133}Cs, ^{138}Ba, ^{139}La, ^{140}Ce, ^{141}Pr, ^{153}Eu, ^{203}Tl, ^{208}Pb and ^{209}Bi) contents using LDA led to a correct identification of nearly 98 % of the samples. Around 100 samples from Asian tea growing areas were included in the study (*12*). Moreover, for selected samples from Nilgiri growing area it was possible to identify the estates.

Neither chemical analysis nor tea tasters are currently able to assess instant teas in terms of origin. It is also not possible to differentiate between an instant green and white tea. The latter is in part due to the lack of an accepted definition.

Based on compositional data for caffeine, theobromine, theanine, and polyphenols, the authenticity of tea products, such as ice teas and instant teas, were analyzed (*13*). Average figures for the analytes in green and black teas were calculated and stated that one third failed to meet the legal limits in selected European countries. It has to be mentioned that for cream treatment in black tea a number of processes are employed (e.g. alkaline treatment) which are known to modify the composition of the instant tea (*1*).

It has to be mentioned that theanine is not a real useful marker for tea authenticity. The theanine concentration is strongly influenced by shading which gives a higher concentration of theanine. Moreover, the stability of theanine in ready-to-drink beverages is not quite clear.

Special Tea Extracts

Due to their use in nutraceuticals, special tea extracts are increasingly sold. There are products on the market since a few years with very high concentrations of catechins (e.g. 60 %). As tea is one of the best natural sources of catechins and contains typical galloylated compounds such as epigallocatechin gallate, authentication of those special extracts is not a problem. The same is true for products containing higher amounts of theaflavins (normally the sum of the major theaflavins does rarely exceed 2.5%). Tea is the only relevant source of theaflavins. The qualification of theaflavins is not a problem due to the characteristic spectra in the visible and UV region and also due to their mass spectra. The quantification might be a problem as theaflavins tend to degrade in diluted solutions. It is also true that no relative response factors as for the catechins have been established. Currently no reference compounds are commercially available to our best knowledge. Consequently, there are not too many data available in the open literature for theaflavin content. There is also no standardized validated method available.

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Coffee Authenticity

What are the relevant issues in coffee authentication? In coffee one of the issues is the detection whether or not Coffea canephora (Robusta coffee) has been used, especially if the on-pack claim states that there is only Arabica in the blend. A German standard method (DIN 10779) uses the determination of 16-O-methylcafestol (16-OMC) to detect *C. robusta* (*14*). According to the available literature 16-OMC is only present in Robusta. The methodology specifies a working range between 50-300 mg kg^{-1}. Unfortunately there was no legal limit set and consequently small amounts (below 50 mg) always make problems. Moreover, some people do treat the concentration of 16-OMC in Robusta samples as constant which is not the case by far. According to early papers by Speer *et al.* (*15*) the amount varies between 680 - 1540 mg/kg, which is a factor of around 2.5. The methodology is kind of tedious but the separation of 16-OMC is quite good. According to Kölling-Speer *et al.* (*16*) this methodology also enables the detection of Robusta in instant coffees which is not within the scope of the original standard method, however, this has not been validated by a ring test as yet. The availability of the reference compound used to be a problem but in the past few years - at least in part due to new isolation procedures (*17*) - this has been overcome. Currently a reference compound is also commercially available.

Numerous other approaches have been published for the same purpose. In coffee currently more than 40 hydroxycinnamic acid esters with quinic acid have been detected and a hierarchical scheme for the identification by HPLC-ESI-MS^n has been published (*18–20*). Hydroxycinnamic acid amides and glycosides have also been found in coffee (*21*). During roasting, the chlorogenic acids are degraded. A part of the chlorogenic acids is converted into lactones (*22*, *23*) which are believed to contribute to the bitter taste of coffee brews (*24*, *25*). In green coffee the chlorogenic acid pattern might serve for the authentication of green coffee (*21*). In that study more than 100 coffee samples (50 Arabicas and 57 Robustas) of known geographic origin were included, and statistical analysis (LDA: linear discriminant analysis and PLS-DA: partial least square discriminant analysis) have been employed for the classification of samples, which was correct in most cases (*21*). This concept will probably not be successful for the authentication of roasted coffee samples or instant products because as mentioned above chlorogenic acids are degraded during roasting yielding breakdown products such as simple acidic compounds and also chlorogenic acid lactones (*23*). If one wants to apply this for roasted coffee samples more data are necessary to check the influence of different roasting conditions. Figure 2 shows a chromatogram of chlorogenic acids and lactones of an instant coffee (Kaiser and Engelhardt 2010, unpublished results).

A comparison of different approaches (elements, chlorogenic acids, and fatty acids) was carried out with respect to Arabica genotypes (traditional vs. ingressed) and terroir. Elements determination gave quite good results in terms of geographic origin but was completely useless in genotype determination, while chlorogenic acids and fatty acids gave around 70-80% correct results for the genotype (*26*).

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A number of papers deal with the detection of geographic origin by stable isotope analysis using IRMS (isotope ratio mass spectrometry) and discriminant analysis. Multivariate analysis of the isotope ratios of the means (δ^{13}C VPDB, δ^{15}N VAIR and δ^{18}O VSMOW) and elemental analysis (carbon and nitrogen) enabled to classify a number of green coffee samples (*27*).

Recently, isoflavones have been claimed to be more abundant in Robusta compared to Arabica, however, the number of samples was very limited in that study (*28*). Especially the content of formononetin was much higher in Robusta coffee compared to Arabica. According to that study the amount of isoflavones decreased during roasting while the extractability increased (*28*).

RAMAN spectroscopy in connection with chemometrics has been employed to assess coffee traceability for Arabica and Robusta samples. The methodology was based on the lipid fraction, namely kahweol. It was claimed that the methodology can be used for the authentication of high grown coffee (*29*).

Near infrared reflectance spectroscopy has also been used for a discrimination of Arabica and Robusta coffees using direct orthogonal signal correction (*30*, *31*). The authors state that not only pure Arabica and Robusta samples can be analyzed but also blends. Another paper also employed NIRS to discriminate between coffee varieties. It was hypothesized that the result ("spectral signature") were affected by environmental factors (*32*).

A special type of coffee (Kona coffee from Hawaii) could be differentiated from other coffees using FTIR-ATR and PLS for both ground coffee and coffee brews (*33*). However, this is a very special application.

A headspace solid-phase microextraction was used as clean-up procedure for a GC-TOF-MS analysis of coffee volatiles to verify the geographic origin of coffee. More than 100 volatile compounds were used for this purpose in combination with statistical analysis; foremost principal component analysis and the result were promising (*34*). One problem mentioned by the authors is the limited stability at least of some of the volatiles. Similar approaches were used by other researchers (*35*) with less volatiles included in the statistical analysis for Arabica and Robusta detection.

Some papers make use of PCR techniques. It has been claimed that PCR-grade DNA can be obtained from roasted coffee as well as from instant coffee samples (*36*). Using PCR-RFLP, adulteration of Arabica with Robusta beans could be detected (*37*).

For soluble coffee authenticity methods do exist. The authentication is accomplished by the determination of carbohydrates, foremost total glucose and xylose (*38*). International standards are in place using HPLC with pulsed amperometric detection (ISO 11292, AOAC method 995.13). More recently, a statistical approach was applied leading to a conclusion that a soluble coffee is adulterated when a value of 2.6% for total glucose and 0.45% for total xylose is exceeded (*39*).

More recently a capillary zone electrophoresis method has been employed for the same purpose (*40*). There is a typical carbohydrate pattern in coffee. Consequently, deviations from that give rise to the assumption that there has been an adulteration.

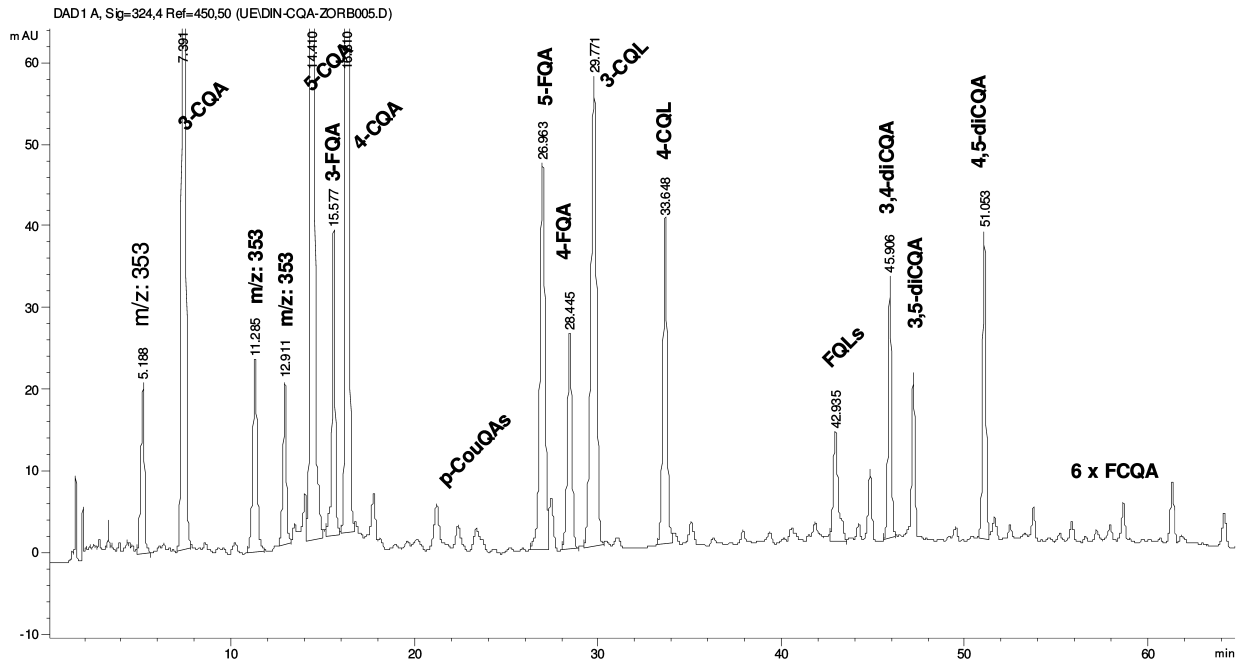

Figure 2. Chromatogram (354 nm) of a roast coffee sample. CQA: caffeoylquinic acid, FQA: feruloylquinic acid, CQL: caffeoylquinic acid lactone, FQL: feruloylquinic acid lactone, FCQA: feruloylcaffeoylquinic acid, CouQA: p-coumaroylquinic acid.

Conclusions

Concepts and analytical methods for the authentication of tea and tea products are available. However, there is still a lack of data for most of the phenolic compounds. The authenticity of special products like white teas is currently a problem. NMR-profiling, SIRA (stable isotope ratio analysis) and minerals have been employed but did not yield generally accepted concepts. Future needs are the use of these methods on one hand and on the other hand accumulation of more data for phenolic compounds to have a more solid basis for the ratio concepts.

For coffee authenticity a number of approaches have been employed. The detection of geographic origin might be more important in the future and consequently a more general accepted method might be useful. To differentiate between Arabica and Robusta samples 16-OMC is a very useful tool. Legal limits would be very helpful if small amounts of 16-OMC are detected.

For both tea and coffee authentication more data are necessary to improve authentication. It is necessary to agree to analytical methods and include those into the routine work in industrial and governemental labs.

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Chapter 17

Evaluation of an FDA Method for the Determination of Melamine and Cyanuric Acid in Food by Liquid Chromatography-Tandem Mass Spectrometry

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A sensitive and selective method was developed and evaluated for the determination of both melamine and cyanuric acid in foods. Foods included pork and fish (edible portions), liquid and powdered infant formula, candy and other low moisture foods. A test portion was extracted with a 50/50 (v/v) acetonitrile/water solution. Proteins were precipitated in the presence of acetonitrile and the supernatant, after centrifugation, was split and cleaned up using two separate solid phase extraction (SPE) cartridges. Mixed mode cation exchange (MCX) SPE was used for melamine and mixed mode anion exchange (MAX) SPE was used for cyanuric acid. All test solutions were syringe filtered and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with hydrophilic interaction liquid chromatography (HILIC) and electrospray ionization (ESI). ESI in the positive mode was used for the determination of melamine and ESI in the negative mode was used for the determination of cyanuric acid. Stable isotopically labeled internal standards ($^{13}C_3$–labeled melamine and $^{13}C_3$-labeled cyanuric acid) were added at the beginning of the extraction procedure and used to

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correct for recovery and any matrix effects. The method limit of quantitation (LOQ) was 20 µg/kg for both melamine and cyanuric acid in pork; 25 µg/kg for melamine and 50 µg/kg for cyanuric acid in fish; and 200 µg/kg for both compounds in dry infant formula and other dry products such as candy. Overall recoveries in spiked test portions in the above matrices ranged from 87-112% for melamine and 78-117% for cyanuric acid. When participating in proficiency testing programs for the determination of melamine and/or cyanuric acid in liquid infant formula, dry milk powder and baking mix, our z-scores were <1.7. A z-score between 0-2.0 was considered acceptable.

Keywords: melamine; cyanuric acid; liquid chromatography-tandem mass spectrometry; solid phase extraction

Introduction

Melamine (1,3,5-triazine-2,4,6-triamine) is produced in large quantities (1.2 million tons in 2007) (*1*, *2*) and primarily used for the synthesis of melamine formaldehyde resin. This resin is used for the production of laminates, plastics, coatings, commercial filters and glues as well as for kitchenware (*2*).

In March, 2007, a pet food manufacturer alerted the U.S. Food and Drug Administration (FDA) to animal deaths linked to certain batches of their pet food. In the following months, consumers and veterinarians reported more illnesses and deaths potentially associated with pet food (*3*–*5*). Further investigation showed that raw materials such as wheat gluten and rice protein were imported from China and used to manufacture pet food (*6*). It appeared that these ingredients were intentionally adulterated with melamine in order to increase their total nitrogen content and consequently, to increase the apparent protein content as measured by the Kjeldahl method (*7*).

In September, 2008, the media revealed severe kidney damage induced by urinary tract stone formation in Chinese infants fed with infant formula and other milk powders tainted with melamine (*1*, *2*). Even though melamine is considered relatively nontoxic, chronic administration of high concentrations can induce renal pathology (*8*). The strong affinity between melamine and cyanuric acid (1, 3, 5-triazine-2,4,6-triol) promoted the formation of the low-soluble melamine-cyanurate complex which was considered responsible for kidney stones (*9*, *10*). Cyanuric acid is either produced as a byproduct during the manufacturing of melamine or by bacteria-mediated metabolism of melamine (*1*). Cyanuric acid is used as a precursor in the synthesis of chlorinated cyanurates which are disinfection products used in swimming pools (*11*).

In October 2008, FDA issued an interim safety and risk assessment stating that levels of melamine and its analogs below 2.5 mg/kg in foods other than infant formula do not raise public health concerns. This assessment had a 10-fold safety factor to compensate for uncertainties regarding combined exposure to more than one of the melamine related compounds. The interim safety and risk assessment

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for infant formula states that levels of melamine or one of its analogues alone below 1.0 mg/kg do not raise public health concerns. This assessment had a 1000-fold safety factor to compensate for uncertainties (*12*).

Considering the presence of melamine in milk and animal feeds and their possible contamination in other food, there is a requirement for establishing sensitive and reliable methods that are capable of screening samples and confirming the presence of melamine and other melamine related compounds. During the period from 2007-2008, FDA developed seven analytical methods for the determination of melamine and/or cyanuric acid in foods. From the seven methods, five were LC-MS/MS methods (*13–17*), one was a GC-MS method, (FDA Laboratory Information Bulletin (LIB) 4423) (*18*), and one was an ELISA method (*19*). A complete summary of most of the analytical methods that were developed during this time period, which includes the above FDA methods, is provided elsewhere (*20*).

The purpose of this paper is to discuss the performance of FDA LIB 4426 (*16*), one of the methods used by FDA field laboratories in recent years, to analyze melamine and cyanuric acid residues in foods such as infant formula, dairy products, pork, fish, and candies.

Summary of Procedure for FDA LIB 4422

The complete details of this procedure are described elsewhere (*16*).This procedure involved the determination of melamine and cyanuric acid by LC-MS/MS in animal tissue and infant formula using stable isotopically labeled internal standards for each compound in order to correct for matrix effects. Prior to the extraction procedure, 250 ng of stable isotopically labeled melamine was added to the test portion, followed by the addition of 2500 ng of stable isotopically labeled cyanuric acid. The forms of the commercially available stable isotopically labeled standards for melamine and cyanuric acid were: $^{13}C_3$ Melamine and $^{13}C_3$ Cyanuric Acid. Tissue samples and liquid infant formula (5 g) were extracted with 20 mL 50-50 acetonitrile-water in a 50 mL polypropylene centrifuge tube. For a 1 g sample of dry infant formula, 4 mL of de-ionized water was first added, followed by 20 mL of 50-50 acetonitrile-water. Extracts were centrifuged and then cleaned up using solid phase extraction (SPE). The cleanup procedure for melamine involved mixed mode cation exchange (MCX) SPE and that for cyanuric acid used mixed mode anion exchange (MAX) SPE. Aliquots of the same extract were individually processed with the two modes of SPE. The MCX SPE was first conditioned with 4% formic acid, followed by loading sample extract and elution of final extract with 2% diethylamine/acetonitrile. The MAX SPE was first conditioned with 5% ammonium hydroxide, followed by loading sample extract and elution with 4% formic acid in acetonitrile. The final extracts for both melamine and cyanuric acid were dissolved in acetonitrile, making the procedure amenable to evaporate the excess solvent for sensitivity needs or solvent exchange. Each compound was analyzed separately using a zwitterionic HILIC LC column (Sequant ZIC-HILIC 5.0 µm; 2.1 mm I.D. x 150 mm) making the analysis time for each compound 30 min. Electrospray ionization was used in

both the negative ion (cyanuric acid) and positive ion (melamine) modes. Two selected reaction monitoring (SRM) transitions were monitored for the native compounds and one SRM was monitored for each stable isotopically labeled compound (Table 1).

Table 1. MS/MS ion transitions monitored

Compound	*Primary Ion Transition*	*Secondary Ion Transition*	*ESI Mode*
Melamine	*m/z* 127→85	*m/z* 127→68	Positive
$^{13}C_3$ Melamine	*m/z* 130→87		Positive
Cyanuric Acid	*m/z* 128→42	m/z 128→85	Negative
$^{13}C_3$ Cyanuric Acid	*m/z* 131→43		Negative

Results and Discussion

The extraction procedure for melamine and cyanuric acid in fish and pork tissue was similar to existing extraction procedures used for extracting melamine in tissue (*13*, *21*) in that 50:50 acetonitrile: water was used as the extraction solvent. The major differences were that the isotopically labeled internal standards were added at the beginning of the extraction procedure, the extract was not acidified and dichloromethane was not used for partitioning, as the partitioning step was not needed due to the ion exchange SPE cleanup procedures.

MCX SPE was used for the cleanup of melamine. Depending on how ion exchange SPE cartridges are conditioned, neutral compounds may be retained on the mixed-mode cartridges. A portion of the tissue extract was loaded onto an MCX SPE conditioned by 4% formic acid in water which allowed for the retention of melamine while the neutral and acidic co-extractives were allowed to pass through the column during the loading and wash steps. Melamine was eluted with a diethylamine-acetonitrile solution. Conversely MAX SPE was used for the cleanup of cyanuric acid. A portion of the tissue extract was loaded onto an MAX SPE conditioned by 5% ammonium hydroxide in water which allowed for the retention of cyanuric acid while the neutral and basic co-extractives were allowed to pass through the column during the loading and wash steps. Cyanuric acid was eluted with a formic acid-acetonitrile solution. The melamine fraction was never combined with the cyanuric fraction because when this was attempted, a precipitate was formed due to the presence of remaining tissue matrix that was not cleaned up using the above SPE procedures. Hence, the fractions were analyzed separately by LC-MS/MS.

Melamine and cyanuric acid were analyzed by HILIC LC-MS/MS. Our experience has shown that due to the high polarity, neither compound is well retained on traditional reverse-phase LC columns (*13–17*). Figures 1 and 2 show MRM chromatograms of extracts of liquid infant formula spiked with 100 µg/kg melamine and 100 µg/kg cyanuric acid, respectively. The chromatograms show

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adequate signals for primary and secondary transitions of both native melamine and cyanuric acid. Experience has shown that the Waters Atlantis HILIC (5.0 µm; 2.1 mm I.D. x 150 mm) can also be substituted for the ZIC® HILIC.

The overall recoveries ranged from 78-117% for pork, fish and liquid infant formula spiked with melamine and cyanuric acid (Table 2). Also, when performing routine analysis on infant formula samples collected from the field, the FDA field laboratories perform spiked recoveries along with each batch of samples for quality control purposes. Each batch of samples was analyzed on different days and the overall recoveries for melamine and cyanuric acid ranged from 80-102% (Table 3).

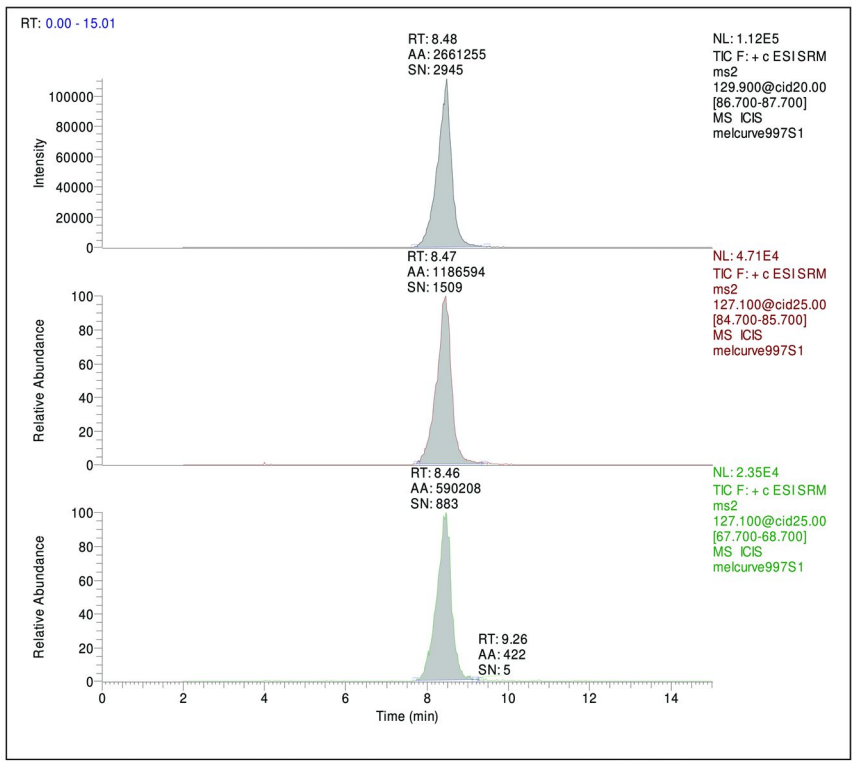

Figure 1. LC-MS/MS chromatogram of 100 µg/kg melamine in liquid infant formula. Top: m/z 130→87 $^{13}C_3$ labeled melamine primary transition; Middle: m/z 127→85 native melamine primary transition; Bottom: m/z 127→68 native melamine secondary transition.

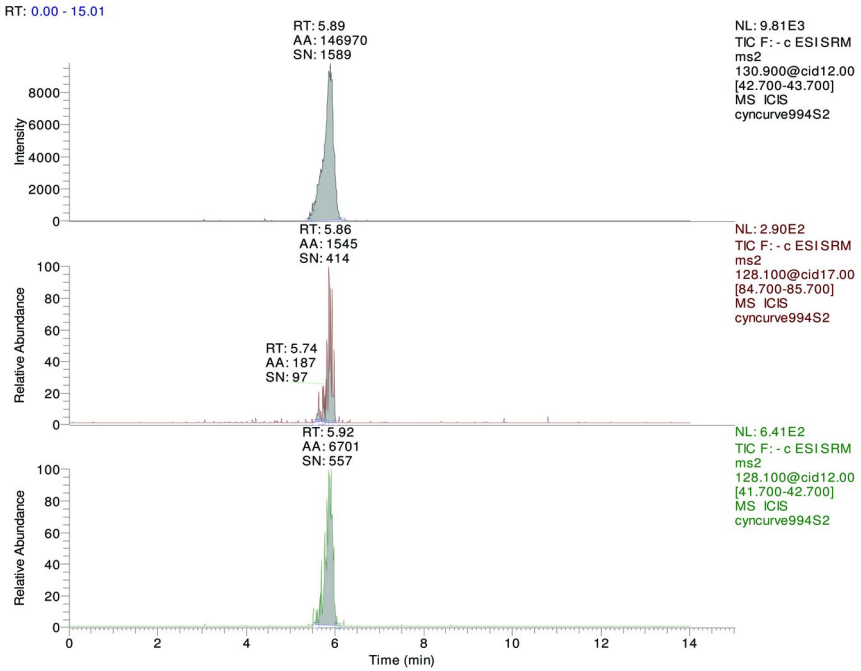

Figure 2. LC-MS/MS chromatogram of 100 µg/kg cyanuric acid in liquid infant formula. Top: m/z 131→43 $^{13}C_3$ labeled cyanuric acid primary transition; Middle: m/z 128→85 native cyanuric acid secondary transition; Bottom: m/z 128→42 native cyanuric acid primary transition.

In addition to fortifying test portions with known quantities of analyte and measuring percent recoveries, it is also important to evaluate how FDA LIB 4422 performs with incurred residues. This can be accomplished by using a reference material (if available), performing analysis of incurred residues between two independent laboratories using the same method, comparing residue values found in incurred residues to those values found by an independent method, and participating in proficiency testing programs. In this study FDA LIB 4422 was evaluated several ways with regard to incurred residues:

First, the analysis of incurred residues of melamine and cyanuric acid in pork and catfish were analyzed by another independent laboratory using the same method. Determination of the incurred residues agreed within 10% for melamine and 20% for cyanuric acid between the two laboratories (Table 4).

Table 2. Recoveries of melamine and cyanuric acid in food

	Melamine			*Cyanuric Acid*		
	Spiking Level (μg/kg)	*Average % Recovery (n=4)*	*% RSD*	*Spiking Level (μg/kg)*	*Average % Recovery (n=4)*	*% RSD*
Pork (Edible Portion)	20	107	8.4	20	108	9.7
	50	86.7	5.7	50	102	3.4
Salmon (Edible Portion)	25	112 (n=6)	5.0	50	117 (n=6)	1.9
	200	95.5 (n=2)	0.74	200	102 (n=2)	1.4
	1000	93.5 (n=2)	0.08	1000	97.0 (n=2)	0.15
Milked-Based Infant Formula (Liquid)	10	97.0 (n=2)	7.2	100	78.0 (n=2)	13.1
	100	102 (n=2)	1.4	500	90.0 (n=2)	4.7

Table 3. Results of recoveries for melamine and cyanuric acid in dry infant formula when analyzed along with field samples for quality control purposes

	Melamine		*Cyanuric Acid*	
Batch No.[a]	*Spiking Level (μg/kg)*	*% Recovery*	*Spiking Level (μg/kg)*	*% Recovery*
1	500	86.8	500	95.2
1	500	89.4	500	101
2	500	102	500	79.5
2	500	98.6	500	94.2
3	500	96.8	2500	99.3
3	500	97.2	2500	102

[a] Each batch represents a different day (n=1 for each replicate).

Table 4. Comparison of results of analysis of incurred residues found in pork and catfish between two independent laboratories using the same method

	Melamine		*Cyanuric Acid*	
Sample	*Lab-1 result (μg/kg)*	*Lab-2 result (μg/kg)*	*Lab-1 result (μg/kg)*	*Lab-2 result (μg/kg)*
Catfish -1	57.0	56.0	<20	<20
Pig Control	20.0	<20	<20	<20
Pig – 1[a]	285	292	1800	2050
Pig – 1[a]	265	287	178	205
Pig – 2[b]	49100	47700	43.0	43.0
Pig – 2[b]	47000	50000	44.0	42.0
Pig – 3[c]	57400	60100	1910	2310
Pig – 3[c]	58100	61200	1970	2360

[a] Pig fed cyanuric acid. [b] Pig fed melamine. [c] Pig fed melamine and cyanuric acid.

Secondly, the results of analyses of a limited supply of samples collected in the field using the FDA GC-MS procedure (*18*) were compared to the results obtained by the LC-MS/MS procedure (Table 5). The laboratory performing the LC-MS/MS method was independent to the one performing the GC-MS method. The two methods were quite different in that the GC-MS procedure did not use stable isotopically labeled internal standards and the extraction procedure for the GC-MS procedure used diethylamine, along with acetonitrile and water. Finally, the GC-MS procedure required first synthesizing the trimethylsilyl derivative for melamine and cyanuric acid prior to the determinative step. The results obtained by these two independent methods agreed within ± 25% (Table 5).

Finally, there was an opportunity to participate in two proficiency testing programs for melamine and/or cyanuric acid in various matrices. The first was a proficiency testing program that involved the determination of melamine and cyanuric acid in liquid infant formula. The concentrations of each analyte ranged from 0.25 – 2.5 mg/kg. The results obtained agreed within ± 10% of the assigned values for melamine and ± 20% of the assigned values for cyanuric acid (Table 6). No Z-scores were assigned with this proficiency test. The second proficiency testing program involved the determination of melamine only in milk powder and baking mix. The assigned value for melamine in milk powder was 10.0 mg/kg and that for melamine in baking mix was 3.18 mg/kg. Our value for melamine in milk powder was 8.7 mg/kg with a Z-score of 1.2 and that for melamine in baking mix was 2.50 mg/kg with a Z-score of 1.6. In this study a Z-score between 0 and 2.0 was considered acceptable.

Table 5. Comparison of results for melamine and cyanuric acid residues from field sample surveys using GC-MS (FDA LIB 4423)[a] and LC-MS/MS (FDA LIB 4422)

	Melamine		*Cyanuric Acid*	
Sample	*GC-MS result (mg/kg)*	*LC-MS/MS result (mg/kg)*	*GC-MS result (mg/kg)*	*LC-MS/MS result (mg/kg)*
Creamy Candy	10.8	9.8	<2.5	<2.5[b]
Creamy Candy	8.1	11.1	<2.5	<2.5
Infant Formula	26.2	27.0	<2.5	<2.5
Infant Formula	19.1	20.7	<2.5	<2.5
Coffee Creamy Candy	<2.5	<2.5	2.9	3.2
Coconut Chewy Candy	<2.5	<2.5	3.3	2.6

[a] The GC-MS analyses were performed by an independent laboratory using FDA LIB 4423. [b] Even though the LOQ for LC-MS/MS is 200 µg/kg for candy and powdered infant formula, the laboratories did not report any values <2.5 mg/kg.

Table 6. Results of analysis of melamine and cyanuric acid residues in liquid infant formula proficiency test samples

Melamine		*Cyanuric Acid*	
Assigned Value (mg/kg)	*Average Values Found (mg/kg) (%RSD)[a]*	*Assigned Value (mg/kg)*	*Average Values Found (mg/kg) (%RSD)[a]*
0.25	0.26 (2.8)	0.25	0.31 (11)
1.00	0.93 (3.8)	1.00	1.17 (3.6)
2.50	2.37 (5.4)	2.50	2.69 (11)

[a] n = 2.

When ESI is used for quantitative analysis, the effect of ionization suppression or enhancement must be considered. The presence of common ions in the sample extract can have a serious suppression or enhancement effect (matrix effect) on the analyte of interest. Improved sample cleanup, matrix dilution, matched calibration standards and addition of a stable isotopically labeled internal standard have been used to minimize and/or eliminate matrix effects. The commercial availability of high purity stable isotopically labeled internal standards for both melamine and cyanuric acid made the internal standard choice the most practical option. Matrix effects were monitored by comparing the response of the primary

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transitions for labeled melamine (*m/z* 130→87) and labeled cyanuric acid (*m/z* 131→43) to the respective responses observed with calibration standards in pure solvent. Signal suppression averaged 40 % for melamine and cyanuric acid in fish, 50 % for melamine and cyanuric acid in pork loin and 35 % for melamine and cyanuric acid in infant formula. These test solutions were sufficiently dilute enough so that matrix effects were not severe (i.e. >60% signal suppression). Despite signal suppression, recoveries were acceptable (78-117 %). The method showed good agreement with incurred residues by independent methods. This suggests that native and labeled primary transitions for melamine and cyanuric acid were equally suppressed due to exact coelution of native and labeled melamine and cyanuric acid from the HILIC column. We used the following criteria to confirm melamine and cyanuric acid in a sample: The ratio of each secondary to primary ion transition for the respective compounds must match within ± 25 % relative to the average value of the corresponding native calibration standards and retention time of suspected analyte peak must match within ± 2 % the average retention time of calibration standards.

The instrumental LOQ is defined as the level at which the ratio of analyte signal to peak-to-peak noise (S/N) is 10:1 for the primary ion transition (*m/z* 127→85) for melamine and (*m/z* 128→42) for cyanuric acid. The LOD is defined as 3:1 S/N for the secondary ion transition (*m/z* 127→68) for melamine and (*m/z* 128→85) for cyanuric acid . The method LOQ is the instrumental LOQ adjusted for the analytical portion's mass and dilution. The method LOQ was 20 µg/kg for both melamine and cyanuric acid in pork; 25 µg/kg for melamine and 50 µg/kg for cyanuric acid in fish; and 200 µg/kg for both compounds in powdered infant formula and other dry products.

Conclusion

A sensitive and specific method was developed for the determination of melamine and cyanuric acid in foods. Even though the original method was developed and validated for tissue, it appeared to perform adequately for the routine analysis of the above compounds in low moisture products such as infant formula. The use of the stable isotopically labeled standards is of importance in order to correct for any matrix effects and recovery loss. Currently, FDA LIB 4422 is routinely used in FDA field laboratories and in other laboratories in the U.S. and other countries. Further work is underway to streamline this method in order to analyze melamine and cyanuric acid in a single chromatographic run.

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Chapter 18

Authentication of Fruit Juices Derived from *Morinda citrifolia* (Noni)

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The consumption of fruit juices derived from *Morinda citrifolia* (noni) has increased tremendously in the last few years especially in North America and Western Europe. This is due to its marketing as a remedy for several complaints. The high price level of such juices makes them prone for adulteration. This may include dilution with water, addition of fruit juices derived from other fruits or addition of sugar as well as the use of concentrated juice instead of direct juice. Another concern is the differentiation between fermented and fresh juice. The limited number of authentic noni juice samples available does not allow the differentiation between the degrees of processing of noni juices for instance with statistical based methods. Thus, a standard analytical method using the neolignans Isoamericanol A and Isoamericanoic acid A as marker compounds has been developed to determine the degree of processing and to ensure the quality of the products.

Introduction

Morinda citrifolia L. (Rubiaceae), commonly known as noni, is an evergreen shrub or small tree. The tree carries oval shaped fruits (10 to 30 cm length) throughout the year. During the ripening process the color of the fruit changes to yellow and almost white, which goes along with a softening of the fruits and the

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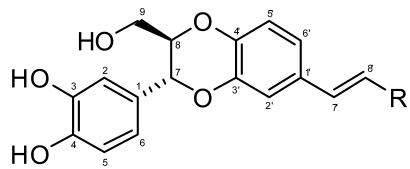
development of an unpleasant rancid cheese-like smell (*1*). Originally, *M. citrifolia* was native to the coastal areas of Southeast Asia (*2*). Two thousand years ago Polynesians disseminated the plant over the Polynesian Islands where the roots, stems, barks, leaves and fruits became a folk remedy against various diseases (*3*).

Since 1996 products derived from noni fruits have been marketed in the United States as a food supplement and in 2003 the European Commission approved noni juice as novel food in the European Union (*4*). The status as novel food implicates that every new noni juice needs a separate approval related to the Novel Food Regulation (*5*). For this approval the assessment of the degree of processing of the juices plays a role that means whether a juice is fermented or non-fermented. Non-fermented juices are simply fresh squeezed juices of *M. citrifolia* fruits whereas for fermented juices the entire fruit with pulp and seeds are stored in containers for 4–8 weeks where a fermentation takes places. During this time, the juice separates from the pulp, which is then pasteurized and bottled (*1*). The storage of the fruits, often described as aging, goes along with an alteration of the flavor (*6*). In this paper, we report the development of a method to differentiate between fermented and non-fermented noni juices using the neolignan compounds shown in Figure 1. These neolignans were previously isolated from plant seeds (*7*, *8*) and lyophilized noni fruits (*9–11*).



	R
Isoamericanol A (**1**)	-CH_2OH
Isoamericanoic acid A (**2**)	-COOH

Figure 1. Chemical structures of the neolignans Isoamericanol A and Isoamericanoic acid A.

Experimental

Chemicals, Solvents

For sample preparation, HSCCC separations, and TLC the analytical grade solvents *n*-butanol, chloroform, dichloromethane, ethyl acetate, methanol, *tert*-butyl methyl ether (Fisher Scientific; Loughborough, UK) and Nanopure water (Barnstead; Dubuque, IA, USA) were used. HPLC-ESI-MS^n and preparative HPLC were carried out with HPLC grade methanol (Fisher Scientific; Loughborough, UK). HPLC grade acetic acid was purchased from Applichem (Applichem GmbH; Darmstadt, Germany).

High-Speed Countercurrent Chromatography (HSCCC)

HSCCC separation of the extracts was carried out on a triple coil *"high-speed countercurrent chromatograph"* model CCC-1000 (Pharma-Tech Research Corporation; Baltimore, MD, USA) consisting of three preparative coils with a total coil volume of 850 mL. Sample injection was done by a manual sample injection valve with 25 mL loop. Solvents were delivered by a Biotronik HPLC pump BT 3020 (Jasco; Groβ-Umstadt, Germany). The UV-absorbance of the eluent was monitored by a K-2501 UV detector (Knauer; Berlin, Germany). The fractions were collected in 4 min intervals into test tubes with a LKB Super Frac 2211 fraction collector (Pharmacia; Bromma, Sweden). The separation was carried out in head to tail mode with a coil speed of 850 rpm and a flow rate of 3 mL/min.

LC-ESI-MS^n

A Bruker HCTultra ETD II LC-MS (Bruker Daltonik; Bremen, Germany) with electrospray ionization in the negative mode was used for the experiments. HPLC system consisted of a HP Series 1100 G1312A binary pump and a HP Series 1200 G1329B ALS SL auto sampler (Agilent; Böblingen, Germany). The system was controlled by Compass 1.3. As dry gas nitrogen with a gas flow of 11 mL/min (350 °C) was used, the nebulizer was adjusted to 60 psi. Following parameters were used: capillary (3000 V), end plate (-500 V), capillary exit (-117 V) and lens 2 (60 V). For the separation a Luna C18 MAX-RP column (250 mm x 4.6 mm, 5 µm particle size, 80 Å pore size) with guard column (Phenomenex; Santa Clara, CA, USA) was used. As solvents (A) 1% acetic acid in water and (B) methanol were used. The screening of the fractions obtained by HSCCC were analyzed using HPLC with the gradient: 0 min 10% B; 20 min 50% B; 45 min 100% B; 55 min 100% B; 60 min 10% B. The quantitation of compound **1** and **2** was carried out using the HPLC-gradient: 0 min 20% B; 5 min 20% B; 15 min 45% B; 25 min 45% B; 35 min 100% B; 45 min 100% B; 50 min 20% B.

Preparative HPLC

For separation of the fraction obtained by HSCCC, a HPLC system (Knauer; Berlin, Germany), consisting of a Smartline pump 1000, a Smartline Manager 5000 containing a low pressure gradient module and solvent degasser, a Wellchrom UV detector K-2600 and a 6-port injection valve equipped with a 200 µL sample loop, was employed. The HPLC system was operated by ChromGate Client/Server Version 3.1.7. Chromatography was carried out on a Luna C18 column (250 mm x 15 mm, 5 µm particle size, 100 Å pore size), protected by a guard column (Phenomenex; Santa Clara, CA, USA). As mobile phases (A) 1% acetic acid in water and (B) methanol were used. Separation of compounds was carried out with the following gradient: 0 min, 40% B; 40 min 70% B; 41 min, 100% B; 45 min 100% B; 46 min 40% B. The flow rate was set to 4.0 mL/min. Absorbance was recorded at 254 nm and 280 nm.

Nuclear Magnetic Resonance Spectroscopy

^{1}H-NMR spectra (600.13 MHz), ^{13}C-NMR spectra (150.90 MHz) and 2D experiments (^{1}H-^{1}H COSY, HMBC, HMQC, NOESY and ROESY) were recorded in CD_3OD on a Bruker Daltonics AVII-600 Spectrometer (Bruker Biospin; Rheinstetten, Germany). The shifts were referenced to the solvent signals at δ_H = 3.31 ppm and δ_C = 49.05 ppm.

Thin-Layer Chromatography (TLC)

TLC was performed using silica gel 60 F_{254} aluminum sheets (Merck; Darmstadt, Germany). Compounds were visualized by spraying with *p*-anisaldehyde/sulphuric acid/glacial acetic acid spray reagent prepared after STAHL and KALTENBACH (*12*). The TLC of the HSCCC fractions was performed using dichloromethane/methanol/water, 75:24:1 (v/v/v).

Plant Material

Fruits of *M. citrifolia* were collected in May 2008 on Tahiti (French-Polynesia), in August 2008 on Hawai'i (USA) and in October 2009 on Cuba. Fruits of *M. citrifolia* from Costa Rica were provided by Wesergold Getränkeindustrie GmbH & Co. KG (Germany).

Preparation of Freshly Squeezed Noni Juice

Fruits were rinsed with water and squeezed with a Para Juice Press (Arauner, Germany). One hundred mL of juice was pasteurized for 1 min at 95°C. The juice was stored at -20°C.

Noni Juice Samples

Authentic noni juice samples were provided from Lifelight Handels GmbH (Salzburg, Austria), Royal Tahiti Noni (Tahiti, French-Polynesia), Healing Noni (Hilo, USA) and S.T. Trading Company (Honokaa, USA). The analyzed seventeen commercial noni juice samples were: Noni 100% Direktsaft (Anton Hübner GmbH & Co. KG, Germany), Cook Islands Noni 100% Direktsaft (GSE-Vertrieb GmbH, Germany), Serrania Bio-Noni 100% Direktsaft (Maneva GmbH Naturprodukte, Germany), 100% reiner Nonifruchtsaft Original (Herbex Ltd., Fiji-Islands), Noni 100% Direktsaft (Golden Heart Products GmbH, Germany), Bio Noni 100% Direktsaft (Sonnenmacht International, Germany), 100% Direktsaft Noni (G&M Naturwaren Import GmbH, Germany), 100% Nonisaft (Allcura Naturheilmittel GmbH, Germany), Noni 100% direct juice (Medicura Naturprodukte AG, Germany), 100% reiner Bio Direktsaft Cook-Islands (Hanoju-Europe Ltd., Netherlands), 100% reiner Noni-Saft Fiji-Islands (Hanoju-Europe Ltd., Netherlands), 100% Noni-Direktsaft Hawai'i (Hanoju-Europe Ltd., Netherlands), Tahitian Noni mixed fruit juice beverage 89% Noni-Saft (Tahitian Noni International, UK), 99.6% Direktsaft aus Nonifrüchten

(Medicura Naturprodukte AG, Germany), Schwechower Noni 27% Noni-Saft (Richard Hartinger, Germany), Bio active 99.6% Noni-Direktsaft (Hirundo Products, Liechtenstein) and Mönchs Noni plus (Mepha GmbH, Germany). The juices were named NJ 1−17. The assignment of the juices was done randomly.

Sample Preparation

Filtered noni juice (10.0 mL) was extracted three times with 20 mL of ethyl acetate. The organic phases were combined, dried with sodium sulfate and concentrated *in vacuo*. The residue was dried in a stream of nitrogen and redissolved in 1000 µL methanol. All samples were filtered through a Teflon micro filter (0.2 µm pore size) before injection into the HPLC.

Extraction and Isolation

Ripe fruits from *M. citrifolia* from Tahiti (3.5 kg) were rinsed with water and squeezed. The seeds were then separated from the pulp and lyophilized to yield 302 g of dried seeds. Noni seeds were crushed and defatted three times with 1.5 L of *n*-hexane overnight. Defatted seeds were extracted three times with MeOH for 24 h. The MeOH phases were evaporated under reduced pressure and lyophilized to give 16.2 g of crude MeOH extract. This extract was redissolved in a 1:3 (v/v) mixture of methanol/water and then successively extracted three times with 1 L of dichloromethane and ethyl acetate each. The collected organic phases were combined, evaporated and freeze-dried to give 2.0 g of dichloromethane extract and 0.8 g of ethyl acetate extract. The entire ethyl acetate extract was separated using HSCCC with ethyl acetate/water, 1:1 (v/v) as solvent system. The extract was dissolved in a mixture of upper and lower phase and injected into the HSCCC. The rotational speed was set to 850 rpm. After 6 h the *elution*-mode was stopped and the *extrusion*-mode was started by pumping 5 mL/min of the upper phase in the coil (*13*). The rotation speed was increased to 1000 rpm. The collected fractions were pooled according to their TLC profiles to give five fractions (F1–5) in the *elution*-mode and additional three fractions (F6–F8) in the *extrusion*-mode. Fractions were analyzed using LC-ESI-MS^n. F8 (140 mg) was further purified by preparative HPLC to give 4 mg of compound **1** and 5 mg of compound **2**.

Results and Discussion

Isolation of the Neolignans Isoamericanol A and Isoamericanoic Acid A

In agreement with previously published work (*14*) and due to the fact that neolignans have been isolated until now only directly from the entire fruit of *M. citrifolia* (*9–11*), we assumed that these compounds are mainly derived from the seeds. Therefore, the seeds of *M. citrifolia* were used as starting material for the isolation of these neolignans.

The isolation was carried out with the all-liquid chromatographic technique of HSCCC after the partitioning of the methanol soluble matter with dichloromethane and ethyl acetate. The neolignan containing fraction was further purified by

preparative HPLC to give the pure compounds **1** and **2**. NMR data is presented in Table I. Structure elucidation was further accomplished by 2D experiments and by comparison with published NMR data (*8*, *11*).

Table I. NMR in CD_3OD data for neolignans 1 and 2

	*Compound **1***		*Compound **2***	
C/H	δ_C *[ppm]*	δ_H *[ppm]*	δ_C *[ppm]*	δ_H *[ppm]*
1	129.58	-	129.43	-
2	115.53	6.85 (*d, J* = 2.0 Hz, 1H)	115.67	6.86 (*d, J* = 2.0 Hz, 1H)
3	146.70	-	147.42	-
4	147.19	-	146.87	-
5	116.35	6.80 (*d, J* = 8.0 Hz, 1H)	116.52	6.81 (*d, J* = 8.0 Hz, 1H)
6	120.43	6.77 (*dd, J* = 2.0, 8.0 Hz, 1H)	120.60	6.78 (*dd, J* = 2.0, 8.0 Hz, 1H)
7	77.65	4.81 (*d, J* = 8.0 Hz, 1H)	77.74	4.84 (*d, J* = 8.0 Hz, 1H)
8	80.12	3.99 (*ddd, J* = 2.5, 4.5, 8.0 Hz, 1H)	80.53	4.05 (*ddd, J* = 2.5, 4.5, 8.0 Hz, 1H)
9	62.14	3.47 (*dd, J* = 4.5, 12.5 Hz, 1H) 3.67 (*dd, J* = 2.5, 12.5 Hz, 1H)	62.16	3.49 (*dd, J* = 4.5, 12.5 Hz, 1H) 3.70 (*dd, J* = 2.5, 12.5 Hz, 1H)
1′	132.09	-	129.62	-
2′	115.65	6.96 (*d, J* = 2.0 Hz, 1H)	117.58	7.17 (*d, J* = 2.0 Hz, 1H)
3′	144.64	-	145.78	-
4′	145.37	-	147.27	-
5′	118.01	6.89 (d, J = 8.5Hz, 1 H)	118.60	6.99 (*d, J* = 8.5Hz, 1H)
6′	120.90	6.93 (dd, J = 2.0, 8.50 Hz, 1 H)	123.29	7.13 (*dd, J* = 2.0, 8.5 Hz, 1H)
7′	131.41	6.49 (*d, J* = 16 Hz, 1H)	145.83	7.55 (*d, J* = 16.0 Hz, 1H)
8′	128.17	6.21 (*dt, J* = 6.0, 16.0 Hz, 1H)	118.15	6.33 (*d, J* = 16.0 Hz, 1H)
9′	63.82	4.19 (*dd, J* = 1.5, 6.0 Hz, 2H)	171.03	-

Analysis of Isoamericanol A and Isoamericanoic Acid A Using LC-MS

The complex matrix of fruit juices derived from *M. citrifolia* required an isolation strategy that allows an enrichment of the target neolignans **1** and **2**. By liquid-liquid partitioning with ethyl acetate, highly polar water-soluble compounds could be eliminated and the neolignans are concentrated in the ethyl acetate extract. The HPLC separation was optimized with different gradients of methanol/water. For the monitoring of the extracts by mass spectrometry the mass range was set to 100−800 *m/z* with the target mass of 350 *m/z*. All measurements were carried out in negative ion mode and the integration of the peaks was done after processing the spectra to extracted ion chromatograms with *m/z* 329 and 343.

For the identification of compounds **1** and **2** in noni juice the HPLC retention times, the masses of the target peaks as well as the fragmentation patterns were compared with those of the isolated reference compounds. Figure 2 shows the chromatograms of the standard compounds **1** and **2** as well as the ethyl acetate extracts of a freshly squeezed juice and a fermented fruit juice. The retention time for compound **1** was 20.7 min and 21.8 min for compound **2**. The MS spectra showed the deprotonated molecular ions of the compounds $[M-H]^-$. These mass-to-charge ratios were *m/z* 329 for **1** and *m/z* 343 for **2**.

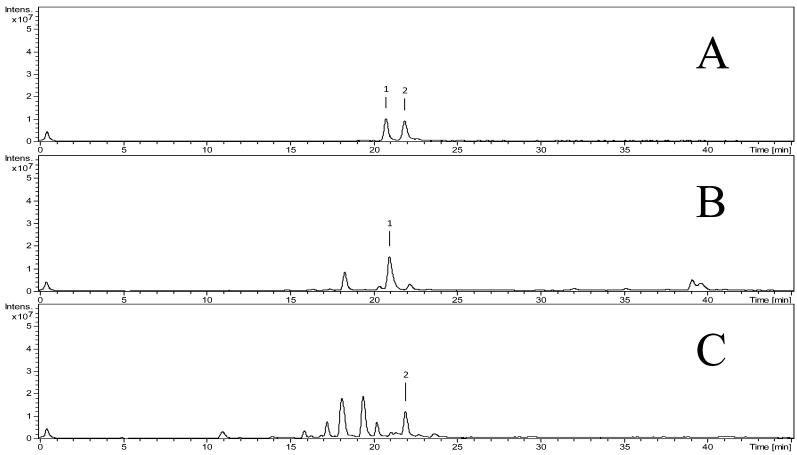


Figure 2. Extracted ion chromatograms of the standard compounds 1 and 2 (A), freshly squeezed noni juice (B) and fermented noni juice (C) with m/z 329 and 343.

Quantitative Analysis of Isoamericanol A and Isoamericanoic Acid A

Quantitative analysis of **1** and **2** in authentic juices and commercial juice samples was carried out using the developed sample preparation and HPLC method. The calibration curves for compound **1** and **2** were obtained with concentrations in five increments. Therefore the peak area was plotted *versus* the injected quantity of the standard solution with linear regression analysis. The

linear regression equation of the compounds **1** and **2** were shown as $y = mx + n$ where x is the concentration and y the peak area of the standard compounds. m and n are constants (Table II). Both curves showed a good linearity with a correlation coefficient > 0.999. The determination of the limit of detection (LOD) and limit of quantitation (LOQ) was carried out according to the DIN method 32645 (*15*). The standard deviation was determined as 5.3 % for compound **1** and 6.0 % for compound **2**.

Table II. Calibration curves, linear ranges, LOD, LOQ and correlation coefficients for 1 and 2 (R = correlation coefficient)

	Calibration equation		*Linear range [mg/mL]*	*LOD [µg/mL]*	*LOQ [µg/mL]*	*R*
	m	*n*				
1	1.018×10^9	-6.396×10^6	0.006–0.041	0.002	0.007	0.9994
2	1.420×10^8	-7.129×10^5	0.005–0.045	0.002	0.005	0.9998

Table III. Contents of 1 and 2 in authentic fruit juices from *M. citrifolia*

Authentic sample	*Compound **1** [mg/L juice]*	*Compound **2** [mg/L juice]*
fermented		
Tahiti 2 weeks	0.84	0.87
Tahiti 8 weeks	n.d.[a]	0.99
Hawai'i 2 weeks	1.47	0.89
Hawai'i 8 weeks	n.d.	0.93
freshly squeezed		
Tahiti ripe	1.87	n.d.
Tahiti unripe	1.71	n.d.
Hawai'i ripe	2.56	n.d.
Hawai'i unripe	2.14	n.d.
Costa Rica ripe	1.35	n.d.
Cuba ripe	1.19	n.d.

[a] n.d., not detectable.

Table IV. Contents of 1 and 2 in seventeen commercial fruit juices derived from *M. citrifolia*

Commercial sample	*Compound **1** [mg/L juice]*	*Compound **2** [mg/L juice]*
NJ 1	n.d.[a]	0.55
NJ 2	n.d.	1.01
NJ 3	< LOQ	2.67
NJ 4	n.d.	2.31
NJ 5	n.d.	0.73
NJ 6	< LOQ	2.44
NJ 7	n.d.	0.53
NJ 8	n.d.	1.01
NJ 9	n.d.	0.86
NJ 10	n.d.	0.54
NJ 11	n.d.	0.83
NJ 12	1.04	1.16
NJ 13	0.92	0.61
NJ 14	n.d.	0.91
NJ 15	n.d.	0.60
NJ 16	n.d.	0.82
NJ 17	1.17	< LOQ

[a] n.d., not detectable.

Initially only ten authentic juice samples were analyzed. Two juices were eight weeks fermented noni juices, two samples were fermented for two weeks, and six juices were freshly squeezed. The contents for **1** and **2** are given in Table III. The values for compound **1** ranged from not detectable to 2.56 mg/L noni juice. Whereas compound **1** could not be identified in the eight weeks fermented juices, the two weeks fermented juices as well as the freshly squeezed juices showed **1** in concentrations from 0.84 to 2.56 mg/L juice. Compound **2** was not detectable in freshly squeezed juices but in all other analyzed fermented juices. The values for the fermented juices ranged from 0.87 to 0.99 mg/L juice.

In Table IV the contents of **1** and **2** in seventeen commercial noni juices are shown. Compound **1** was detected in five of the seventeen juices from 0.92 mg/L to 1.17 mg/L. Two juices showed values below the limit of quantitation (LOQ). Compound 2 could be detected in all of the seventeen analyzed noni juices. One juice showed only traces of **2**. The values of the other analyzed commercial juices ranged between 0.53 and 2.67 mg/L juice. Taking the results of the authentic juices into consideration, the two juices NJ 12 and NJ 13 which exhibited significant

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concentrations of compounds **1** and **2** were medium fermented juices. The juice NJ 17 which revealed only compound **1** was assumed to be a freshly squeezed juice.

Conclusion

For the determination of Isoamericanol A and Isoamericanoic acid A in noni juice an HPLC method has been developed. By using this method it is possible to distinguish freshly squeezed and fermented noni juices. Isoamericanol A is characteristic for freshly squeezed juices, whereas Isoamericanoic acid A could serve as a marker for fermented juices. This investigation provides a screening method to evaluate the degree of processing and could be useful for the quality control of noni juice products. The oxidative formation of acid **2** is the subject of ongoing research.

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Chapter 19

Authentication of Fruit Juice Aroma: Evaluating Re-Aromatization

Effects on Apple Juice Aroma during Production and Storage

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The re-aromatization of apple juice from apple juice concentrate can be evaluated on the basis of an aroma index. This is a complex formula considering the contents of several aroma compounds that are typical for apple juice aroma. Changes in aroma during production and storage must be considered when evaluating fruit juice aroma. The numerous technological processes in the course of the production of apple juice from concentrate are sources for several losses in the aroma content. For example, during aroma recovery, mixing, and filling a considerable part of the total aroma is lost. The aroma content also decreases dramatically during storage. The degradation of aroma occurs in all different kinds of packaging materials, although with certain differences.

Introduction

Fruit juices belong to the most regulated and defined products in the food industry. Numerous methods and parameters have been established for evaluating fruit juice authenticity. The analytical assessment of characteristic components like sugars, acids or mineral nutrients gives information about fruit juice content and possible adulterations. Therefore, the analytical results are compared to established data. This comparison is done in view of the natural ranges of

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variation. Reliable data bases are compiled inter alia in the AIJN-Guidelines and Code of Practice (*1*) or the Codex Alimentarius (*2*).

In the field of aroma compounds the determination of the ratio of enantiomers has proven to be an important and meaningful analytical technique to uncover addition of artificial or chemically synthesized flavor. Numerous chiral compounds occur in juices and fruit products. These substances, e.g., the esters ethyl 2-methylbutyrate and 2-methylbutyl acetate, are naturally found only in specific enantiomeric ratios, as the forming enzymes work very specifically. In contrast chemical synthetic routes often yield racemic product mixtures (*3*, *4*).

A new field of analytical activity in the evaluation of fruit juices is the evaluation of the amounts of special aroma compounds. These are used to evaluate aroma content, degree of re-aromatization, and aroma quality. Several private food control laboratories and consumer advice centers try to link aroma quality of fruit juices to the absolute aroma content. The absolute content of the selected flavor compounds is used to evaluate re-aromatization, which is directed for juices from concentrate by European law (*5*).

Apple Juice Aroma

Up to now more than 300 volatile compounds have been identified in apples and apple juice (*6*, *7*). These substances contribute more or less powerfully to the typical aroma impression of apple juice. As far as quantity is concerned, the substance classes of esters, aldehydes, and alcohols dominate the aroma profile of apple juice. Also diverse substances with low odor threshold values contribute significantly to the typical apple juice aroma (*8*). Among these inter alia ?-damascenone and acetaldehyde shall be emphasized as these compounds intensively contribute to the total aroma impression.

Apple juice is a natural product and its composition varies in broad ranges. Numerous influencing factors cause this broad range of variation. Besides the main parameters, like brix and acidity, the constellation of aroma compounds, forming the typical apple juice aroma, is strongly influenced, too (*8–12*). Considerable differences occur both in the qualitative and in the quantitative composition of flavor compounds. Variety is probably the most decisive factor.

The qualitative aroma composition differs within the varieties (*13*). There are varieties in which the aroma is dominated by esters of acetic acid, whereas other varieties mainly form esters of 2-methylbutyric acid or also other acids. Some variety-dependent differences are listed in Table I for juices of the varieties Gehrers Rambour, Remo, and Oldenburger. The juice of the variety Remo contains high amounts of esters of acetic acid - especially n-propyl acetate, 2-methylbutyl acetate and hexyl acetate. The juice made of Gehrers Rambour-apples contains only traces of acetates but high amounts of ethyl 2-methylbutyrate and ethyl butyrate. The juice of the variety Oldenburger contains only small amounts of esters. The varieties have been analyzed at different degrees of ripeness. Table I shows the mean values of the analyses at different states of ripeness.

Some other aroma compounds like aldehydes and some alcohols (e.g., hexanol, *(E)*-2-hexenol) are formed immediately after destruction of the cell

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structure in all varieties. The amounts of these typical compounds of apple aroma depend on the activity of the involved enzyme systems (*14*, *15*).

The aroma content is also influenced by climate, geographic origin, and time of harvest. These factors primarily influence the quantitative composition (*10*).

The amounts of aroma compounds change during storage and are also influenced by storage conditions (i.e., temperature). At first, the concentrations of special substances especially the esters increase, whereas during prolonged storage their amounts decrease.

Table I. Comparison of the contents of some selected aroma compounds of juices of the apple varieties Gehrers Rambour, Remo, and Oldenburger

	Gehrers Rambour	*Remo*	*Oldenburger*
Esters of acetic acid			
Ethyl acetate	35	716	93
n-Propyl acetate	0	1134	0
2-Methylbutyl acetate	2	2739	19
Butyl acetate	0	2956	10
Hexyl acetate	6	1331	0
Esters of butyric acid and 2-methylbutyric acid			
Ethyl butyrate	1348	5	20
Methyl 2-methylbutyrate	7	33	1
Ethyl 2-methylbutyrate	370	26	9
Hexyl 2-methylbutanoat	6	99	26
Selected alcohols and aldehydes			
2-Methylbutanol	2855	3648	6927
1-Hexanol	1158	1736	1417
(E)-2-Hexenol	701	380	383
Hexanal	1155	415	1062
(E)-2-Hexenal	5258	4343	4277

NOTE: All units are g/L

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Parameters for the Evaluation of Apple Juice Aroma

Because of the broad variability in the qualitative and quantitative composition of apple juice aroma, the development of pertinent AIJN-reference guidelines for single aroma compounds is not feasible (*11*). These guidelines would have to cover such wide ranges that no useful information would be attained.

Sum parameters may be an useful alternative. They offer a good capability to compensate specialities and anomalies in the aroma composition. Lacks in the contents of some flavor compounds can be compensated by high amounts of others. Suggested models for the quantitative evaluation of apple juice aroma are the Sum of esters (SoE), the Sum of carbon-6-aldehydes and -alcohols (SoC6) and the Aroma Index (AI) (*10*, *14*).

Sum of Esters

Esters contribute significantly to the apple juice aroma. They smell fruity and have mostly low odor threshold values in water and apple juice. Because of their high volatility they are well suited to characterize the aroma impression and to assess re-aromatization. The SoE is a classical sum parameter proposed for the evaluation of apple juice aroma by Heil and Ara (*14*). It includes the aroma compounds ethyl isobutyrate, methyl 2-methylbutyrate, ethyl 2-methylbutyrate, ethyl butyrate, 2-methylbutyl acetate, hexyl acetate, hexyl 2-methylbutyl acetate, ethyl hexanoate, ethyl 3-methylbutyrate, and 3-methylbutyl acetate. Butyl acetate a ester, that occurs in apple juice in high amounts, too, is omitted because otherwise its high concentrations would dominate the parameter. The contents of the particular substances are summed up to a total.

Sum of Carbon-6-aldehydes and alcohols

The SoC6 is a classical sum parameter as well. It includes hexanal, *(E)*-2-hexenal, hexanol and *(E)*-2-hexenol. These substances convey the green character, which is just as elementary for the typical apple juice aroma as the fruity aroma impression mediated by the esters. The majority of the alcohols and aldehydes has significantly higher odor threshold values than most of the esters. However, they are usually present in higher amounts and therefore do strongly contribute to the overall apple juice aroma.

A disadvantage of these two sum parameters is that both approaches sum the individual aroma substances without weighting. Because of this, some substances that occur in higher amounts may dominate the parameter, while compounds that contribute to the aroma in even lower contents are discriminated or disregarded.

Table II. Standardization Factors of the Aroma Index Model

Aroma Compound	*Standardization Factor*
Ethyl 2-methylbutyrate	2.000
Ethyl butyrate	0.500
Hexyl acetate	0.333
2-Methylbutyl acetate	0.250
Butyl acetate	0.100
Hexanal	0.100
(E)-2-Hexenol	0.050
(E)-2-Hexenal	0.025
Hexanol	0.017
2-Methylbutanol	0.033

Aroma Index

The so-called Aroma index is a weighted sum parameter (*10*, *16*). The contents of the ten included substances (A_1-A_{10}) are summed with consideration of standardization factors (f_1-f_{10}). These factors equalize differences in the scale of contents. This prevents that substances that are present in higher amounts dominate the parameter. Each compound is weighted equally as 10% of the total aroma.

Aroma Index (AI) = $A_1*f_1 + A_2*f_2 + ... + A_{10}*f_{10}$

The standardization factors (Table II) are derived from reference values which have been generated out of a statistical database (*16*). The reference values form the lower guideline that has to be reached in order to obtain an apple juice of average aroma content.

The AI contains ten substances (cf. Table II). Out of the SoE, the substances ethyl 2-methylbutyrate, ethyl butyrate, 2-methylbutyl acetate, and hexyl acetate are taken into account. Additionally, the four components of the SoC6 together with butyl acetate and 2-methylbutanol are considered. These ten substances have been identified as typical apple juice aroma compounds. They contribute in view of their usual contents in apple juice and their low odor threshold values in water and apple juice - significantly to the typical apple juice aroma (*9*, *11*, *16*).

Correlation of the Sum Parameters with the Organoleptic Impression

Up to now no explicit correlation of one of the sum parameters presented above with the organoleptic impression has been proven or evidenced. The parameters may convey a general overview with regard to the aroma intensity, but the aroma quality can not be derived from any sum parameter, that has been suggested yet.

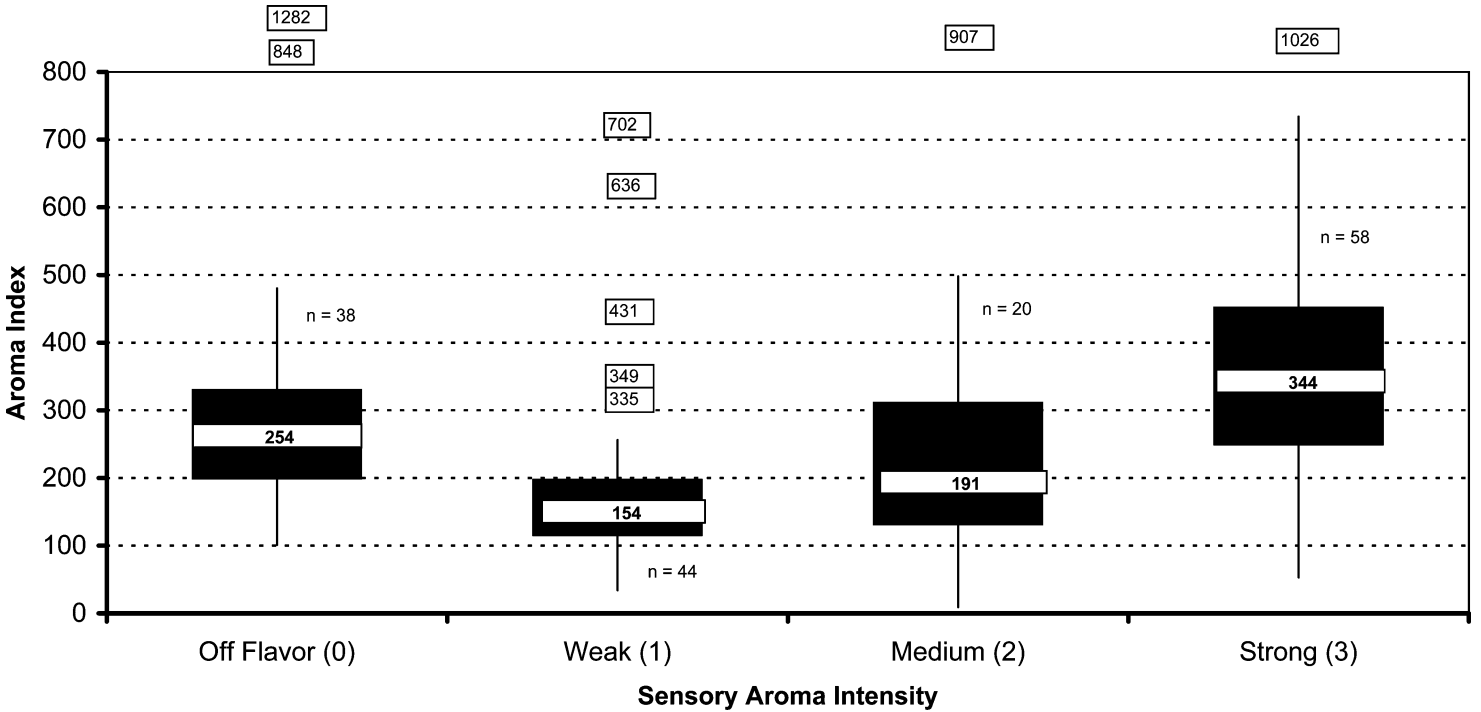

Figure 1. Correlation of the Aroma Index Model with the sensory aroma intensity. Numbers in boxes are possible outliers, n is the number of samples pooled in each intensity class and the number in the middle is the median value. The panel consisted of five skilled panelists.

The correlation of the organoleptic impression with the AI is shown in Figure 1 with a box-and-whisker-plot. One hundred sixty apple restoration aromas (natural water phases that are recovered during the production of apple juice concentrate) have been evaluated organoleptically and categorized into the four classes Off-Flavor, Weak, Medium and Strong intensity. The box-and-whisker-plot proves that the AI is definitely applicable for the evaluation of aroma intensity. While the median of class 1 (weak intensity) is 154, it is clearly higher at approximately 191 in class 2 and 344 in class 3. However, it should be noted that a considerable number of water phases of medium or high intensity (classes 2 and 3) show an AI of less than 100 always applied to the content in the restored juice (in assumption of a standard dosage of 1:150). On the other hand aromas with really high flavor contents often displayed stale off-flavors.

Consequently, aroma analysis as a sole criterion does not provide information about the organoleptic quality of an aroma. It merely has the function to indicate whether certain aroma substances are typical for the respective juice. The sum parameters can be used to verify general re-aromatization. However, aroma quality is mainly a matter of sensory evaluation.

Changes and Losses during Production

When fruit juice aroma is evaluated quantitatively, it must be considered that the aroma undergoes substantial changes during production and storage. The numerous technological processes in the course of apple juice production from apple juice concentrate offer multiple possibilities for losses in the aroma content, e.g., during aroma recovery, mixing, and filling.

Losses during Aroma Recovery

A concentration equipment with linked aroma recovery plant is depicted in Figure 2. By using this device, several stage control tests were carried out in order to determine the recovery rate of the evaporated aroma.

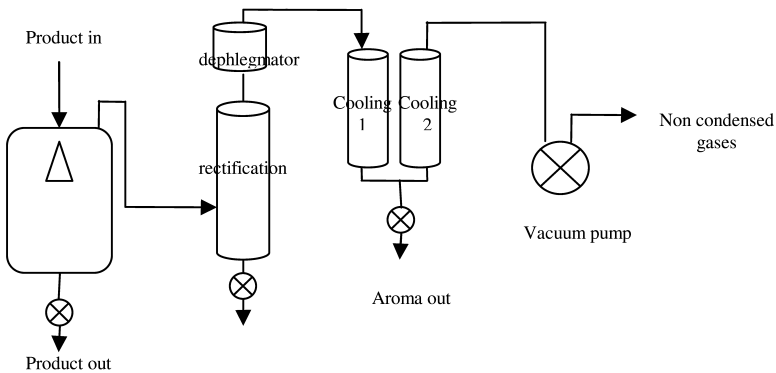

Figure 2. Aroma recovery plant

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Table III. Recovery rates achieved in stage control tests on an aroma recovery plant

	Test period I	*Test period II*	*Test period III*
SoE	66 %	62 %	74 %
SoC6	94 %	82 %	100 %
Aroma Index	77 %	68 %	80 %

The course of the trials is subdivided in three test periods with varied settings (Table III). Although numerous parameters have been optimized, a recovery rate of only 74 % for the highly volatile esters could be achieved. Less volatile substances could be recovered to a higher degree than the highly volatile ones. The SoC6 were recovered to nearly 100 % (see Table III).

As decisive factor in optimizing the recovery rate the temperature of the cooler has been discovered. Lower cooling temperatures yielded enhanced recovery rates. An addition of a gas washer at best with liquid aroma as fluid also improves the recovery rate. The typical aroma compounds of apple juice are easily volatile so that they are vaporized even at low exhaust steam rates (*17*). They can easily be removed from the juice and are not detectable in the fruit juice concentrate.

Losses during Mixing and Filling

Due to highly automated and optimized machinery, the losses in mixing and filling are well controlled and minimized. Nevertheless some losses could be detected. This is mainly during the process step of degassing. Juices need to be degassed before filling to eliminate dissolved gases like oxygen that otherwise might disturb the filling process or affect the product and shorten shelf life. The commonly used procedure for degassing is the vacuum-ventilation-method. Four different plants have been examined with regard to losses in highly volatile aroma compounds. The four degassers differed slightly in temperature, vacuum pressure, and volume. These parameters have been adjusted empirically for best filling conditions.

On average 10% of the highly volatile aroma compounds (determined as the sum of esters) are lost during filling. That means a comparatively small loss, but has to be considered as well.

Changes and Losses during Storage

Numerous storage trials showed that the aroma content of apple juices decreases dramatically during storage. The decrease in flavor compounds has been observed in all kind of tested packaging material including glass, polyethylene terephthalate (PET), and cardboard packaging (Tetra).

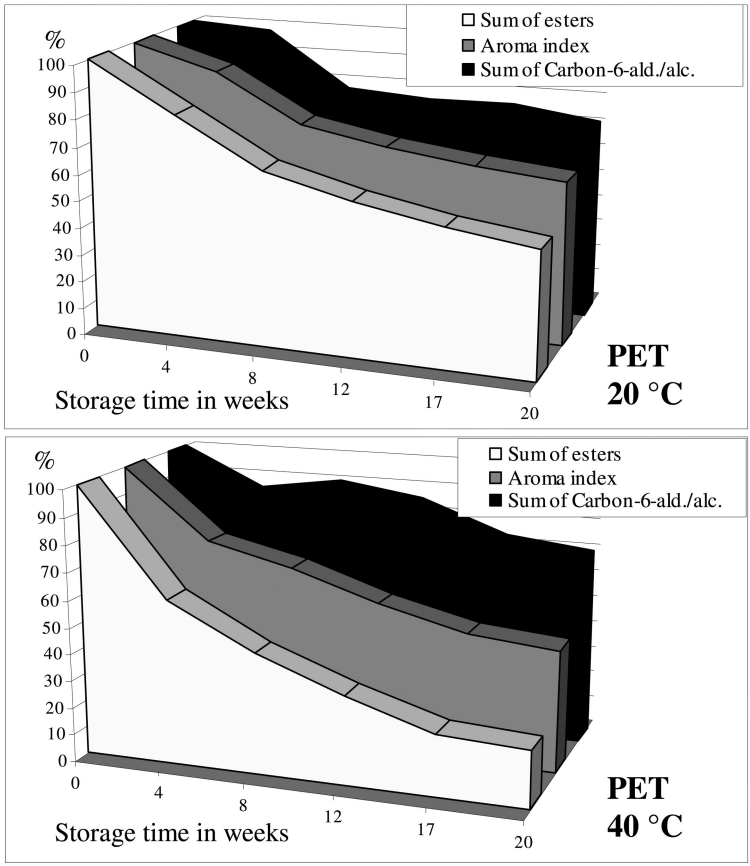

Figure 3. Storage of apple juice from concentrate in PET for 20 weeks at 20 C and 40 C.

The changes in aroma content during storage have been followed with the sum parameters SoE, SoC6, and AI. At regular intervals the amounts of the flavor compounds have been determined and compared to the initial content after filling.

All sum parameters showed a clear decline over storage time. The decline of the SoE was more drastic than that of the SoC6. The AI which is based on both substance groups was in the middle. The breakdown of aroma substances, especially the esters, was highly temperature dependent (Figure 3). At 40 C the decline proceeded 2-4 times faster than at 20 C.

After storage for 20 weeks at 20 C, the relative content of the SoE went down to 47 %. At 40 C a relative content of 45 % was already observed after 8 weeks. After 20 weeks at 40C the SoE declined to 21 %. In contrast temperature had a minimal effect on the loss of SoC6 during storage. After 20 weeks relative contents of 71 and 70 % respectively, were attained.

Similar courses of degradation were observed during storage trials in cardboard packaging and glass bottles, as well. In Tetra packaging the relative content of the substances summed in the SoE declined to 45 % within 30 weeks

at 20 C. At 40C this content was already achieved after 12 weeks. The amount of the substances combined in the SoC6 decreased at both temperatures to approximately 80 %.

Changes in the Sum of Carbon-6-aldehydes and alcohols

As mentioned above the the amount of the SoC6 declined in the average by 20-30 % during 4-8 months of storage at 20C.

A closer look into the sum parameter revealed that not all of the compounds summarized in this parameter reacted the same. On average all included compounds showed a decline, but the contents of the aldehydes hexanal and *(E)*-2-hexenal decreased faster than those of hexanol and *(E)*-2-hexenol. This was more evident at 40 C than at 20 C. For the breakdown of hexanal and *(E)*-2-hexenal diverse pathways have been postulated (*18*). The mainly oxidative reactions result in the formation of several acids (e.g. butanoic acid, 2-hexenoic acid, 3-hexenoic acid), aldehydes (e.g. 3-hydroxyhexanal, 3-ethoxyhexanal), and isomerization products (e.g. *(Z)*-2-hexenal, *(E/Z)*-3-hexenal) (*18*).

The higher transformation rates of the aldehydes lead to a shift in the percentages of the SoC6 (Figure 4). The proportions of alcohols especially hexanol increased.

Changes in the Sum of Esters

The SoE declined drastically during storage. Figure 5 shows a summary of the diverse storage trials in all three packaging materials with the accompanying linear regressions. The trend lines were used for better illustration of the overall trend.

On average only 40% of the initial ester content remained in the juice after 12 months of storage at 20 C. This trend was observed for all the different kinds of packaging material investigated.

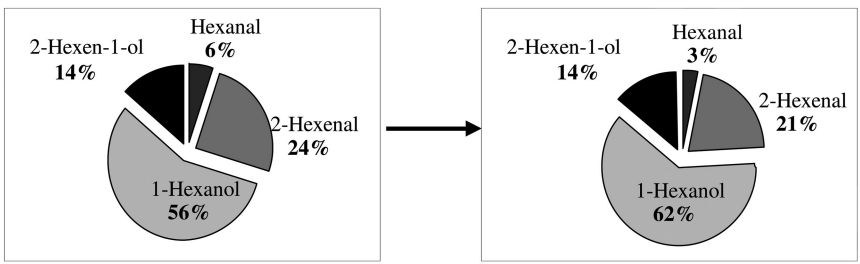

Figure 4. Changes in the percentages of the SoC6 during storage.

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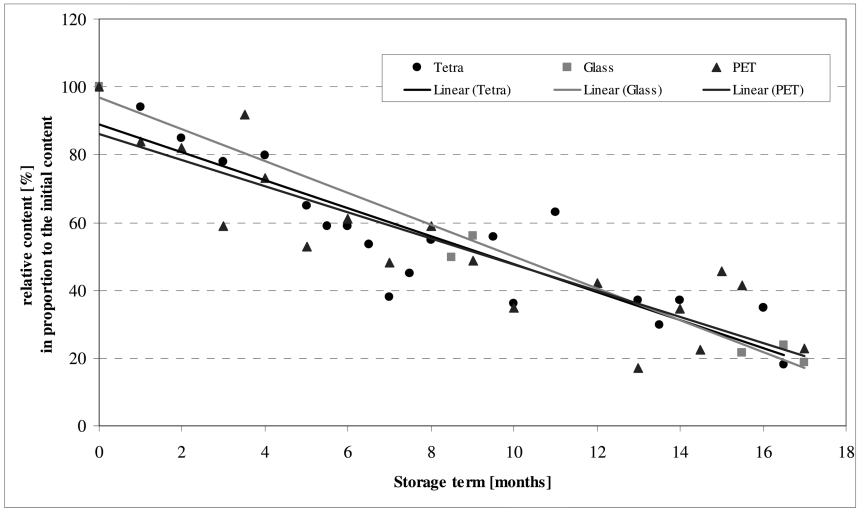

Figure 5. Changes of the SoE during storage in different kinds of packaging materials.

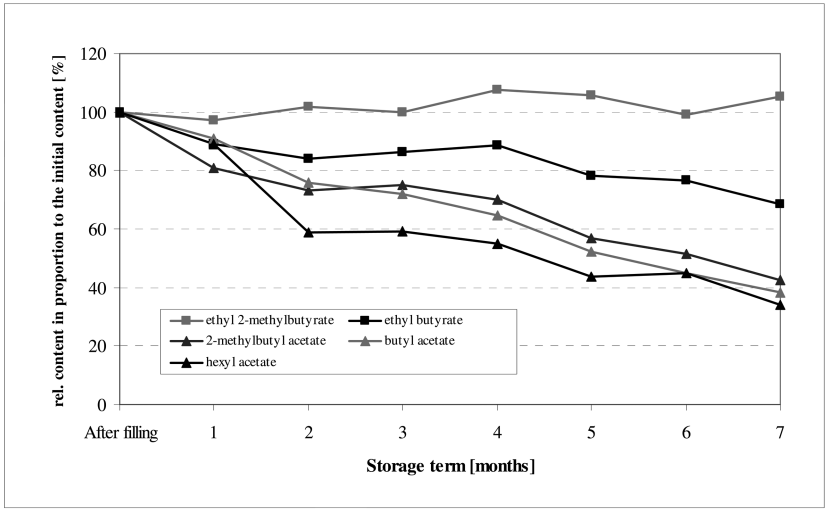

Figure 6. Changes in the contents of individual esters.

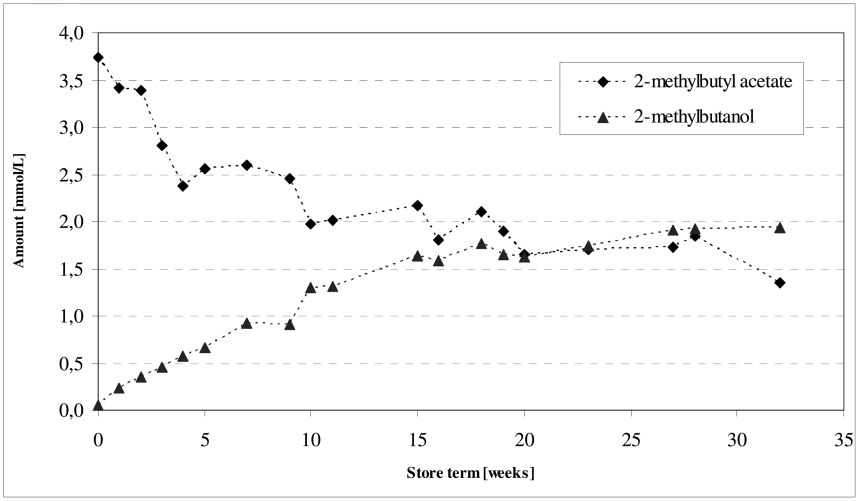

Figure 7. Changes of administered 2-methylbutyl acetate during storage in model matrix.

Like the components of the SoC6, not all esters contained in the SoE reacted exactly the same. In our trials the acetates (ester of acetic acid) showed a faster decline than the esters of butyric acid and 2-methylbutyric acid.

Figure 6 shows an example for the decline of the individual esters in apple juice. The esters of acetic acid 2-methylbutyl acetate, butyl acetate, and hexyl acetate showed a more drastic decrease over storage time than ethyl butyrate and ethyl 2-methylbutyrate. After six months the amount of 2-methylbutyl acetate, butyl acetate, and hexyl acetate decreased to 45-50% of the initial content after filling. The amount of ethyl 2-methylbutyrate stayed quite constant and the content of ethyl butyrate was reduced by 20 %.

As an opposite effect to the decrease of esters the contents of acetic acid and 2-methylbutyric acid rose. This suggests an acid-catalyzed ester hydrolysis. But in the complex mixture of natural apple juice no definitive statements to stoichiometric developments can be uttered. Moreover, the acids are polar substances and can not be reliably determined by simultaneous distillation-extraction (SDE), the choosen preparation method.

Storage Trials in Model Matrix

To throw light upon possible reactions of the esters during storage, storage trials have been carried out using a model matrix. An apple juice concentrate that was confirmed to be free of volatile aroma compounds was diluted to 11,2 brix, the usual brix of clear apple juice from concentrate. To this diluted juice definite amounts of aroma compounds were added and their concentration measured during 32 weeks of storage in glass bottles at 20 C. The pH of the model solution was 3.5-3.6, the one of a natural apple juice.

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Figure 7 shows the results of the storage trial of 2-methylbutyl acetate in the model matrix. 486 mg/L of 2-methylbutyl acetate were added to the apple juice basis. Over storage time at 20 C a rapid decrease occurred which was similar to the degradation rates determined in apple juices. Simultaneously, the amount of 2-methylbutanol increased. The molar amounts of the substances in mmol/L suggest a nearly stoichiometric reaction.

Hence it seems that the esters, although considered quite stable under acid conditions, can undergo acid hydrolysis in juices. This hydrolysis leads to a decline in the SoE over storage time.

Changes of the Aroma Index

As the SoE clearly goes down during storage time and the SoC6 undergoes several changes as well, the AI changes during storage, too. Figure 8 shows the summary of diverse storage trials in all three packaging materials with the accompanying linear trend regressions. The AI declined with storage time. Only little outliers to higher values were observed. All trend lines showed nearly corresponding gradients. For the trials in glass higher values were detected. This might be connected to the better oxygen barrier qualities of glass and therefore smaller losses in the SoC6 in glass. In general, after 12 months approximately a quarter of the amount of compounds summarized in the AI was lost.

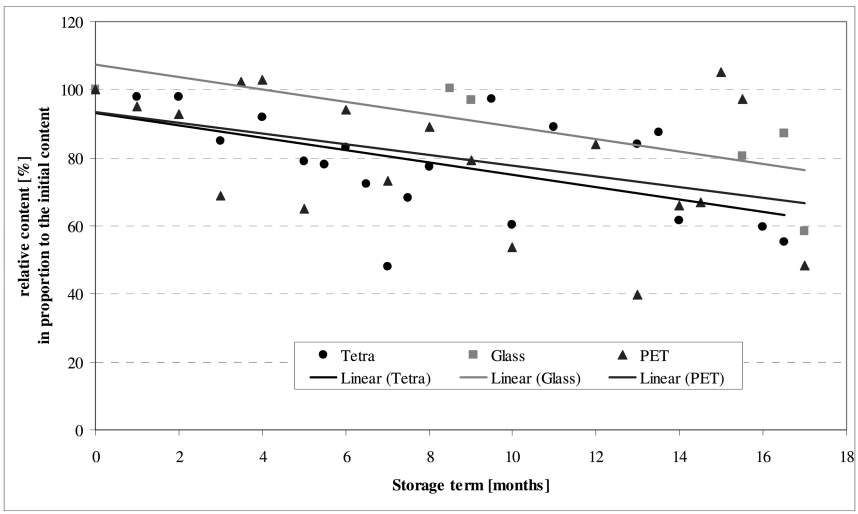

Figure 8. Changes in the aroma index during storage.

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Conclusion

Since apples have wide variations in their aroma content, guideline values for individual aroma compounds of apple juice are not feasible. Better suited are sum parameters, which are more independent of varietal specialties and enable a better authentication of back-added apple juice aroma. This is especially true for the aroma index model which combines the models of the sum of esters and the sum of carbon-6-aldehydes and alcohols and uses weighted parameters.

In the quantitative evaluation of fruit juice aroma and of the re-aromatization of fruit juices from fruit juice concentrate the unevitable losses during production have to be taken into account. Such losses have been determined during processing and filling. Furthermore the aroma content decreases drastically during storage. After 12 months approximately a quarter of the initially contained aroma is lost. The amount of the sum of esters declines by approximately 60 % over one year of storage at 20 C.

These losses during production and storage have to be considered in the expert evaluation of fruit juice aromatization and especially re-aromatization of fruit juices from concentrate. Depending on storage time and conditions reduced contents of aroma may be reasonable and do not unconditionally indicate missing re-aromatization.

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Chapter 20

Rapid Authentication of Fruit Juices by Infrared Spectroscopic Techniques

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Authentication of fruit juices is a major challenge for an industry that is fast growing and expanding. Traditional methods to monitor authenticity such as HPLC, GC or sensory methods are time consuming and require well-trained personnel. There is a need for a rapid, easy to use, inexpensive method to monitor the authenticity of fruit juices. Infrared technology has gained acceptance as a rapid and reliable technique. It can be used to evaluate the overall sample composition, providing a fingerprint that is characteristic of the product. This eliminates the need to test for several individual components. Multivariate statistical analyses are used to evaluate the infrared spectral information. This technique could be used to determine addition of foreignmaterials, differentiate different juice commodities, or even to evaluate geographical origin of a juice.

Background

The fruit juice market has increased considerably over the last decades (*1*). Some of the driving forces behind this growth are the increased demand for natural products and ingredients and the push from health organizations across the world for increased consumption of fruits and vegetable products.

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Fruit juices have gained momentum as the most competitive segment in the beverages industry with more people around the world incorporating fruit juices in their meals amidst rising awareness of maintaining healthy and nutritious eating habits. Fruit juice consumption per capita is highest in the US, Canada and Japan (*1*), as well as the European Union. However, fruit and vegetable juice consumption is growing rapidly around the world, particularly in Asian countries, including China and India. The juices consumed in largest volumes are orange, apple and grape, followed by pineapple and grapefruit (*2*, *3*).

As seen in Table 1, citrus juices, such as orange juice, have had a clear dominance over the juice marketplace. However, regardless of the lower consumption levels, grape juice still manages to be a multi-billion dollar industry. This is due to the high value associated with grape products, considered the most valuable crop among fruits, nuts, and vegetables. This value is likely due not only to the specific sensory attributes of juice cultivars, but also their associated health benefits.

Table 1. U.S. per capita consumption of selected fruit juices in liters per year single strength. (adapted from (*3*))

Season	*Apple*	*Grape*	*Pineapple*	*Cran-berry*	*Prune*	*Total Non-Citrus*	*Total Citrus*	*Total Fruit Juice*
1990	6.53	1.06	1.90	0.53	0.15	10.18	19.80	29.98
1995	5.97	1.71	1.44	0.61	0.11	9.84	23.33	33.17
2000	6.84	1.29	1.14	0.76	0.07	10.11	22.61	32.72

Fruit juices contain mainly water, considerable concentrations of carbohydrates, other solids, acids, and low levels of enzymes, pigments, flavor compounds, and lipids. Many fruit juices (Figure 1, Table 2) have been shown to be a rich source of polyphenols (*4*), a class of compounds known for their antioxidant activity. This antioxidant activity not only has a protective effect on the food matrix it exists within, but carries the benefit on to the tissues of the consumer as well (*4*). As a source of polyphenols, Concord grape juice ranks high among other grape juices (Figure 1).

The composition of Concord grape juice is very unique compared to similar fruit juices and grape juices from other varieties. Sugar content, acidity, and most notably phenolic content of Concord grape juice are distinctive and consistent. This unique composition is responsible for Concord grape juice's characteristic sensory quality as well as the specific health benefits associated with its consumption. Both of these factors give Concord grape juice increased value and make it a more likely target for adulteration with lower cost juices. Interestingly, it is Concord grape juice's unique composition that allow for it to be chemically discriminated from other juices.

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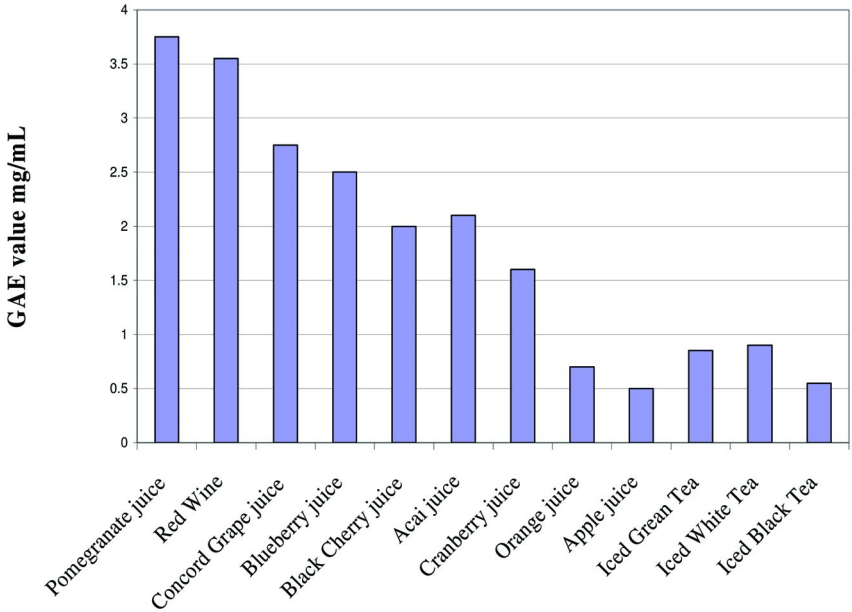

Figure 1. Phenolic content (gallic acid equivalents, GAE) of common polyphenol rich ready-to-drink beverages (5).

Grapes of different varieties and geographical origins have vastly different prices, but as of yet it is difficult to differentiate them analytically. This vulnerability leaves juice manufacturers susceptible to fraudulent behavior from suppliers and competition. This is such an important concern because it would potentially result in economic loss as well as consumer deception due to mislabeling. Moreover, recent studies have outlined the potential health benefits of Concord grapes, making their detection even more desirable.

FT-IR spectroscopy has gained acceptance as a method for the rapid identification of compounds, due to the fact that they yield highly reproducible spectra. Foods can generate a unique and characteristic absorbance spectrum, or "fingerprint," based on the vibrational modes of the compounds it contains. This would allow for the determination of juice composition based on FT-IR analysis coupled with statistical classification. Multivariate statistical software collects the information from over hundreds of spectra and further conducts data reduction analysis, known as Principal Component Analysis (PCA), in order to extract information from the training data set. Importantly, this allows for the construction of statistical models capable of predicting the identity and content of future unknown samples.

Table 2. Typical composition of some common commercial juices. Degree Brix represents percent sugar

Juice	*°Brix*	*Total Polyphenols mg GAE/mL*	*Polyphenolics*	*Source*
Apple	11.5	0.4 ± 0.1	Proanthocyanidins, epicatechin, quercitin, catechin, myricitin	(*5*, *6*)
Blueberry	10	2.3 ± 0.4	Proanthocyanidins, anthocyanins (Mv, Dp, Cy, Pt and Pn derivatives), quercetin, epicatechin, myricetin	(*5*, *6*)
Cranberry	7.5	1.7 ± 0.2	Proanthocyanidins, anthocyanins (Cy, Pn derivatives), quercetin, myricetin, catechin	(*5*, *7*)
Grape	16	2.6 ± 0.4	Proanthocyanidins, anthocyanins (Mv, Dp, Pn, Cy derivatives), myricetin, quercetin, catechin	(*5*, *8*)
Grapefruit	10	1.2 ± 0.1	Naringenin, hesperetin	(*6*, *9*)
Orange	11.8	0.7 ± 0.1	Hesperetin, naringenin, quercetin, myricetin	(*5*, *6*)
Pomegranate	16	3.8 ± 0.2	Anthocyanins (Dp, Cy, Pg derivatives), gallic acid, ellagic acid	(*5*, *10*)
Tomato	5	0.8 ± 0.1*	Quercetin, kaempferol, myricetin	(*6*, *11*)

Cy:cyanidin; Pg:pelargonidin, Dp: delphinidin, Pn: peonidin, Mv: malvidin, Pt: petunidin. * Ferulic acid equivalents, mean of 13 commercial tomato juices.

Fruit Juice Authentication

Food and juice adulteration has long been a problem for the food industry. In fact, the original Food and Drug Act of 1906 made it "unlawful for any person to manufacture within any Territory or the District of Columbia any article of food or drug which is adulterated or misbranded" (Federal Food and Drugs Act of 1906 Public Law Number 59-384 34 Stat. 768). Unfortunately, this problem persists to this day due to unsuitable methods for detection of adulteration at the production scale. In addition, replacement of high value ingredients with lower grade, cheaper substitutes such as sugars or low cost juices such as white grape or pear, can be an attractive and lucrative temptation for unscrupulous food manufacturers or suppliers of raw materials.

US law considers a food to be adulterated if an ingredient or component has been omitted or substituted, if damage or inferiority of the product has been concealed, or if a substance has been added to increase bulk or strength or to make it appear of greater value (Food Drug and Cosmetic Act, section 403B).

Two main strategies have been traditionally used to determine juice adulteration. The first is to look for foreign compounds, evidence of the presence of extraneous material. The second approach is to evaluate for expected concentrations and/or proportions of natural components (*12*). Evaluation of a single component is often not sufficient information to determine authenticity, therefore analyses of multiple components is typically required (*13*). For example, while the presence of over 10 ppm of naringin in orange juice is considered evidence of added grape juice, both high sorbitol and proline levels in apple juice, provide evidence of pear juice addition (*12*).

Currently, chromatography is the most commonly used method of adulteration detection and is often coupled with mass spectroscopy detection, but these methods require extensive extraction and purification steps (*14*). Liquid chromatography, gas chromatography and capillary gas chromatography are examples of the techniques more frequently used. However, chromatographic methods are time-consuming, wasteful of organic solvents, and require a trained technician (*15*). Sensory tests require panelists to be consistently trained and are not always accurate when used for discrimination (*16*).

There are other less common means of authentication that have been investigated. Electronic noses have been used to evaluate volatiles, this method is rapid but still not accurate enough for discrimination (*16*). Analysis of stable carbon isotope ratios requires "databases for certified authentic products" to be effective (*17*). Pyrolysis mass spectroscopy, coupled to multivariate analyses has been reported to have considerable potential as a rapid method to determine food authenticity, although it has so far received little attention (*13*).

FT-IR spectroscopy monitors the entire chemical composition of the sample as opposed to individual compounds, making monitoring subtle compositional differences more practical. Other advantages of FT-IR spectroscopy as a technique for food authentication include rapid and simple analysis, qualitative and quantitative results, no harmful solvents, non-destructive of sample, highly resolved spectra, and simple instrumentation (*18*), all essential qualities for a method of determination to be used routinely by manufacturers (*19*). Multivariate

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data reduction and pattern recognition techniques have been used in conjunction with FT-IR spectroscopy in order to authenticate a wide range of products. Because of this, FT-IR coupled with multivariate analysis is ideally suited for monitoring and authenticating complex food matrices.

Infrared Spectroscopy

Infrared spectroscopy studies the interaction of infrared light with matter (*20*). The IR region consists of near infrared (NIR, 10000-4000 cm^{-1}), mid infrared (MIR, 4000-400 cm^{-1}), and far infrared (FIR, 400-10 cm^{-1}). When IR radiation strikes a substance, it can be transmitted, reflected or absorbed. As absorption takes place, the chemical bonds exhibit vibration, stretching, bending or contracting motions. Specific group of atoms in the molecules (known as "functional groups") tend to absorb radiation from the same spectral area (*20*). Since the absorption will be observed in the same range, regardless of the molecule the functional group is in, it is possible to correlate the range of absorption of any given sample with its chemical structure (Table 3). As a result, the sensitivity of IR spectroscopy to the presence of functional groups in matter can be utilized for identifying unknowns, confirming identities and quantifying concentrations (*20*). The number, frequencies, intensities, and half-widths of the IR absorption bands represent a highly characteristic fingerprint of a given compound and no two structurally different molecules, with the exception of optical enantiomorphs, possess the same IR spectrum (*24*). Furthermore, the IR spectra are not only qualitative, but also give information about the quantity of functional groups (*25*).

The use of the absorption information obtained with an interferometer by Fourier transform infrared (FT-IR) spectroscopy improves spectral reproducibility and wavenumber precision in comparison to results from dispersion instruments (*26*). Advantages of FT-instruments include collection of reproducible spectra through the internal use of a fixed wavelength laser as a standard (Connes' advantage).

FT-IR uses larger slits than normal monochromator units meaning that there is higher radiation throughput and therefore better sensitivity, known as the Jacquinot advantage. Finally, because all wavelengths of IR radiation are detected simultaneously, a large number of spectra can be sampled and averaged which enhances the signal to noise ratio (Felgett's advantage). FT-IR spectroscopy monitors the entire sample chemical composition making monitoring subtle differences more practical and ideally suited for monitoring and authenticating complex food matrices.

The infrared spectra of foods are very complex and often require mathematical transformation to extract relevant information (Figure 2). Mathematical transformations of spectral data are used to enhance the qualitative interpretation of the spectra.

Table 3. Tentative band assignment in FT-NIR and FT-MIR spectral frequencies (cm^{-1}) of juice extracts. Based on data from (*15*), (*21*), (*22*), and (*23*)

FT-NIR (cm^{-1})	*Assignment*
7270	combinations of CH vibrations
6900	first overtone stretching modes of free OH
6645	first overtones of NH stretching modes
6963 and 4823	O-H overtones/combination bands in crystalline sucrose
5950-5700	first overtone of C-H stretching
5800 and 5685	C-H overtone modes of aliphatic groups in lipids
5200	O-H combination band
4850 and 4600	combinations of amide A/I and amide B/II bands, respectively
4400- 4033	C-H and C-C overtone and combination bands of carbohydrates
4450-4340	C-H overtones of carbohydrates
4428	O-H bending / hydrogen bonded O-H stretch
4393	O-H stretch / C-C stretch
4340 and 4261	C-H combination bands of aliphatic groups of of lipids
MIR (cm^{-1})	***Assignment[a]***
3600 – 3000	O-H stretching and H-O-H bending of water
3500	νOH stretching

Continued on next page.

Table 3. (Continued). Tentative band assignment in FT-NIR and FT-MIR spectral frequencies (cm^{-1}) of juice extracts.

MIR (cm^{-1})	*Assignment*[a]
2930 – 2800	C-H asym/sym stretching of CH_3 and CH_2
2959	ν_a (CH_3) stretching of methyl
2921	ν_a (CH_2) stretching of methylene in fatty acids
2872	ν_s (CH_3) stretching of methyl
2858	ν_s (CH_2) stretching of methylene in fatty acids
1732, 1726	C=O stretch; unconjugated ketone and/or carboxyl group
1715	ν (C=O) stretching of esters, carbonic acids
1655	amide I band
1660-1640	C=C vibrations; aromatic system and C=O stretch; conjugated ketone and/or quinones
1548	amide II band
1600-1400	C-C stretching, benzene ring of aromatic skeletal, and C=C-C stretching; aromatic ring, and O-H plane bending of alcohols
1515	"tyrosine" ring vibration
1498	"phenylalanine" ring vibration
1468	δ (CH_2) bending of methylene
1400	ν_s (COO^-) stretching of carboxylates
1367	C-O stretching; ester groups and primary alcohols

MIR (cm^{-1})	*Assignment*[a]
1310-1240	amide III
1250-1220	ν_a (PO_2^-) stretching of phosphodiesters
1084-1088	ν_s (PO_2^-) stretching of phosphodiesters
1200-900	C-O-C, C-O dominated by polysaccharides ring vibrations
1242, 1165	C-O stretching; primary alcohols (sugars)
976	C-O, C-H, C-C and C=C vibrations in carbohydrates, aromatics
720	ρ (CH2) rocking of methylene
1200-600	Fingerprint Region

[a] s=symmetric, a=asymmetric, ν=stretching, δ=deformation, ρ=rocking.

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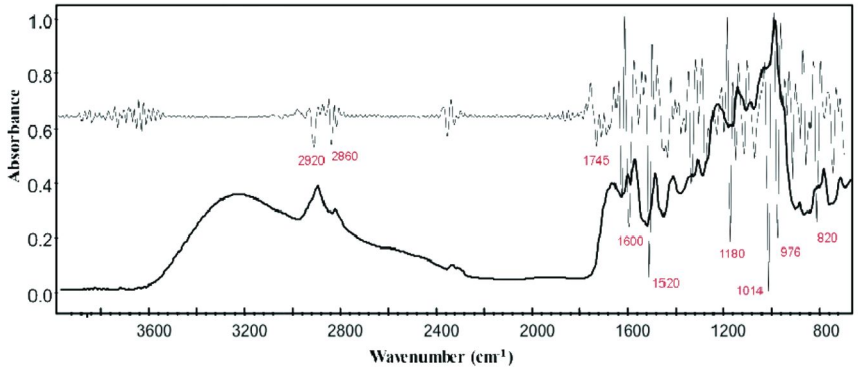

Figure 2. Fourier transform mid-infrared (4000-700 cm^{-1}) raw spectra (A) and vector-length normalized and second derivative transformed (Savitzky-Golay, five-point window) spectra (B) of the phenolic-fraction of anthocyanin-containing juices using a 3-bounce ZnSe-ATR crystal.

The standardization of the spectra by using smoothing and SNV pre-processing steps improves the signal-to-noise ratio of the data and corrects for the nonlinear light scattering effects of the samples and baseline offsets (*27*). The Savitzky-Golay second derivative transform allows the extraction of useful band information (Figure 2) through the removal of baseline variations and resolution of overlapping peaks (*28*, *29*).

FT-IR spectra contain so much data that extracting useful information from them may require the application of multivariate data reduction and pattern recognition techniques. In order to create an effective method for monitoring and authenticating any number of food products, including fruit juices, a large number of samples must be analyzed. Lavine (*30*) highlights the need for data analysis techniques that allow for analysis of huge data sets, much like those typical of IR spectra, as well as allow for creation of relationships based on "similarity or dissimilarity among groups of multivariate data" instead of traditional quantitative terms. These similarity-based comparisons are perfectly suited for authentication and adulteration monitoring. Downey (*31*) discusses the food processing industry's need for rapid, inexpensive analysis "to verify the nature of high-value foods" especially, and reports the use of principal component analysis (PCA), a multivariate analysis technique, on infrared spectra to detect honey adulterated with beet invert syrup and high fructose corn syrup. Principal component analysis allows for the compression of data. This is especially helpful in chemical data sets that generally contain redundant data preventing efficient data analysis. Redundant data are highly correlated data that are generally less useful. An infrared spectrum, for example, contains a great deal of highly correlated data because the generated spectra are continuous in shape and each data point is correlated to other nearby points. Principal component analysis is capable of eliminating this redundancy by taking the measurement variables of a spectrum (absorbance vs. wavenumber) and creating a single principal component axis for comparison.

Multivariate statistical methods can be divided into unsupervised and supervised techniques. Unsupervised methods analyze data without prior knowledge of the samples, grouping data into clusters having similar attributes (i.e., hierarchical cluster analysis or HCA) or finding linear combinations of the original independent variables that account for maximum amounts of variation (i.e., principal component analysis or PCA) (*32*). In supervised methods, on the other hand, each spectrum is assigned to a definite class, so qualitative data is added to the quantitative spectral data (*33*). Examples of supervised methods are discriminant analysis, canonical variate analysis, and soft independent modeling of class analogy (SIMCA). SIMCA has been used with great success to classify a number of fruit juices based on FT-IR spectroscopic data (*15*). SIMCA is a PCA-based approach to modeling classes and provides a means of classifying samples into groups. In SIMCA, samples described by spectra are classified as single data points, reducing the data to a manageable level. SIMCA is especially useful in chemical studies where the number of variables exceeds the number of samples. Most models would have issues of collinearity, but SIMCA can work with as few as 10 samples per class (*30*). Partial Least Squares (PLS) analysis provides a "quantitative multivariate modeling method (*34*)." An effective PLS model uses a training data set to make predictions about samples based on other data about that sample, such as a FT-IR spectrum. PLS has been used to model physico-chemical properties of pomegranate and grape juice concentrates such as titratable acidity and total soluble solids as well as to predict the level of adulteration of pomegranate juice with grape juice concentrate (*35*).

Application of Infrared Spectroscopy in Juice Authentication

The need for a rapid means of food authentication and the disadvantages associated with traditional methods to effectively classify samples leaves the food industry, and potentially the consumer, vulnerable to not only fraudulent behavior, but also to unsafe products. FT-NIR and FT-MIR have been evaluated as rapid and reliable tools for authentication and detecting adulteration in fruit juices (Tables 4 and 5).

Fourier-Transform Near-Infrared Spectroscopy

FT-NIR absorption spectroscopy is a very attractive technology for process/quality control because it for allows fast, accurate and non-destructive measurements of chemical components and can provide information about structural and physical properties of materials (*36–38*). NIR absorption spectroscopy is based on the relatively weak and broad overtones and combination bands of fundamental vibrational transitions associated mainly with C-H, N-H, and O-H functional groups (*39*). The NIR bands are 10-100 times less intense than the corresponding mid-infrared fundamental bands enabling direct analysis of samples that are highly absorbing and strongly light scattering (i.e, contain small particulates) without dilution. NIR measurements of aqueous systems have been challenging because of the interference from broad vibrational bands of water

(*40*). However, the strong effect of water signals (Figure 3) can be minimized by rapid solvent elimination and measuring the dry extract by diffuse reflectance spectroscopy (*41*)(*42*), by using the derivatized spectra on transmittance (1 or 0.5 mm cell cuvette) or by transflectance (1 mm pathlength) measurements (*22*, *43*). Models generated from transmittance spectra have shown the best performance statistics and accurately and precisely predicted sugar levels in non-scattering juices while NIR transflectance data improves the prediction errors for scattering juice samples (*43*).

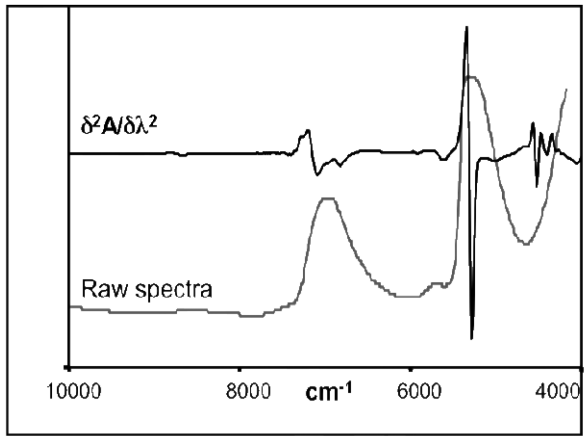

Figure 3. Raw and second derivative (37 pt. size) spectra of sugar solutions using transmittance FT-NIR technique (22).

NIR spectroscopy has been proposed for quality monitoring in the sugar industry (*44*) and for the detection of juices, purees and syrups adulterated with cheaper juice concentrates, cane, corn or beet sugar/syrups. Twomey et al. (*45*) reported the use of NIR and factorial discriminant analysis for the detection of adulteration of orange juice with orange pulpwash, grapefruit juice, and synthetic sugar/acid mixture. Accurate classification rates, >90%, were determined for adulterated orange juice at 50 g/kg or higher levels, with no adulterated orange juice being predicted as authentic. The use of PLS-1 algorithms based on the near-IR spectral data of orange juice extracts dried on fiberglass filters (*42*) has allowed for determination of glucose, fructose, sucrose, and citric acid with an acceptable precision (coefficient of variation for validation, CV% ≤ 10%) (*41*). Contal et al. (*46*) showed that adulteration of strawberry or raspberry juice with apple juice could be detected at levels >10% by using PLS-NIR models. FT-NIR spectroscopy in combination with discriminant (Linear discriminant analysis and canonical variate analysis) and quantitative (Principal component regression and Partial least squares regression) analysis has been successfully applied for the classification of adulterants (pure and invert cane and beet syrups) in maple syrup (*47*).

Table 4. Application of Infrared Spectroscopy for Classification of Fruit Juices

Juice	*Target*	*Method*	*Chemometrics*	*# of samples tested*	*% Accuracy*	*Reference*
Apple	Alicyclobacillus spp.	NIR	PCA/SIMCA	NA	89%	(*57*)
	Content of actual juice	MIR	Potential Curves	Varied by concentration	100% at lower conc.; 71-100% at higher concentration	(*58*)
	Discrimation of *E. coli* 0157:H7 from other *E. coli* strains	MIR	PCA/SIMCA	30 spectra	82% compared to *E.Coli* ATCC 25522	(*59*)
	Adulteration with HFCS, sugars or both	NIR	Discriminant PLS regression	450 pure and 600 adulterated	96% for adulterated and lower value of 86% for authentic	(*31*)
	Adulterantion with PICS, BS, HFCS, FGS	ATR-IR, 11 reflections	PLS & k-nearest neighbors	75 authentic, 240 adulterated	96.5 (PICS), 93.9 (BS), 92.2 (HFCS), and 82.4% (GFS).	(*31*)
	Differentiation by heat treatment	MIR/NIR	PLS-2 and LDA	105	77.2%	(*60*)
	Differentiation by variety	MIR/NIR	PLS-2 and LDA	105	78.3-100%	(*60*)
Bayberry	Variety Discrimination	Vis-NIR	PLS and BPNN	60	100%	(*61*)
	Water adulteration	NIR	PC-RBFNN	NA	97.6%	(*62*)
Mango	Added sugar content	ATR-MIR	PLS	165	100% with detection limit of 3.6% added sugar	(*63*)

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Table 4. (Continued). Application of Infrared Spectroscopy for Classification of Fruit Juices

Juice	*Target*	*Method*	*Chemometrics*	*# of samples tested*	*% Accuracy*	*Reference*
Orange	Source authentication	NIR	PCA	92	100% with 25 PC's	(*2*)
	Differentiation of commercial samples	HATR-IR	Discriminant function analysis	7 not-from-concentrate, 3 from-concentrate	Excellent cluster separation	(*54*)
Pomegranate conc. (PC)	Adulteration with grape juice concentrate	ATR-IR	PCA	PJC with added 2-14% grape concentrate (v/v)	99% differentiation between PJC and GJC	(*35*)

PICS = partially inverted cane sugar; BS = beet sugar; HFCS = high fructose corn syrup; FGS = glucose, fructose and sucrose solution. NIR = Near infrared spectroscopy; MIR = Mid infrared spectroscopy; NA = Data not available; PCA = Principle component analysis; SIMCA = Soft independent modeling of class analogy; PLS = Partial least squares discrimination; LDA = Linear discriminant analysis; PC-RBFNN = Principle component scores for radial basis function neural networks; BPNN = Backpropagation neural networks.

Table 5. Application of Infrared Spectroscopy for Quantitative Analyses of Fruit Juices and Products to Monitor Authenticity

Product	*Target*	*Method*	*Results*	*Reference*
Apple juice	Adulterants: PICS, BS, HFCS, FGS	ATR-IR, 11 reflections	SECV PICS = 4.9%; BS = 4.6%; HFCS = 4.6% and FGS = 9.5%	(*31*)
Apricots	Soluble Solids	NIR	SEP = 0.8	(*64*)
	Citric Acid	NIR	SEP = 0.08	(*64*)
	Flesh firmness	NIR	SEP = 4.9	(*64*)
Bayberry Juice	Acids	Vis/NIR	SECV = 0.19	(*65*)
Black currants	Soluble Solids	MIR	RMSEC = 0.53Brix	(*66*)
	Total Acidity	MIR	RMSEC = 1.69 g/L	(*66*)
Japanese pear juice	Pectin	NIR	SEP = 1.41	(*67*)
Mango juice	Soluble Solids	MIR	SEP = 1.10 with r(2) = 0.99	(*63*)
Orange juice	Tartaric Acids	Vis/NIR	SEP = 0.013	(*61*)
	Citric Acid	Vis/NIR	SEP = 0.596	(*61*)
	glucose, fructose, sucrose, citric and malic acid	NIR	SEP (g/L) glucose = 1.57, fructose = 1.54, sucrose = 3.02, citric acid = 0.91, malic acid = 0.21	(*41*)
Tomato juice	Lycopene	ATR-IR, 3 reflections	SECV = 0.5 mg/100g	(*68*)

Continued on next page.

Table 5. (Continued). Application of Infrared Spectroscopy for Quantitative Analyses of Fruit Juices and Products to Monitor Authenticity

Product	*Target*	*Method*	*Results*	*Reference*
Tomato products	beta-carotene	Raman/NIR	SECV = 0.16	(*69*)
White Grape Juice	Soluble Solids	MIR	SECV = 0.20 Brix	(*70*)

PICS: partially inverted cane sugar; BS: beet sugar; HFCS: high fructose corn syrup; FGS: glucose, fructose and sucrose solution. Vis: UV/Visible Spectroscopy; NIR: Near infrared spectroscopy; MIR: Mid infrared spectroscopy; SEP: Standard error of prediction; RMSEC: Relative mean standard error of prediction; SECV: Standard error of cross-validation.

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Models developed from FT-MIR spectra, by using the carbohydrate, organic acid and amino acid fingerprint regions have resulted in models with superior discriminative performances for detecting fruit juice adulterants as compared to those obtained from dispersive NIR spectra (*31*). Similar results have been reported for the analysis of thyme, oregano and chamomile essential oils by dispersive NIR and ATR-MIR spectroscopy (*48*).

Mid-infrared (MIR) spectroscopy monitors the fundamental vibrational and rotational stretching of molecules, which produces a unique chemical profile of the sample. While sample preparation can be time consuming for some types of MIR techniques, it has become trivial by using Attenuated Total Reflectance (ATR) instruments. In ATR devices, the incident IR beam is separated from the sample by a special crystal fabricated with infrared transparent materials that have specific refractive indexes. When the light hits the crystal it forms an evanescent wave that slightly penetrates, and is "attenuated" by, the sample. Typical depth of penetration varies from 0.5 to 5 microns (*20*). Since only very small amounts of sample are required, and greater quantities will not affect the spectral results, ATR allows the IR analysis of a wide variety of materials, in a rapid, non-destructive manner, qualities that are essential for a method of determination to be used routinely by manufacturers (*19*).

FT-MIR has seen use in label claim verification of honey, authentication of pomegranate juice concentrate, discrimination of red wine cultivars, and juice authentication (*15*, *35*, *49*, *50*). Multivariate analysis has found use in authentication and adulteration testing in such commodities as orange juice and tomatoes (*51*, *52*). Furthermore, FT-MIR spectroscopy coupled with multivariate analysis has been studied for detecting adulterations in honey and pomegranate juice (*35*, *49*). In grapes it has been shown that differences in the composition of numerous flavan-3-ol monomers provide the best indication of varietal differences (*50*). Fourier transform infrared spectroscopy and attenuated total reflection sampling was used to detect adulteration of single strength apple juice samples with partially inverted can syrup adulterants, beet sucrose, or high fructose corn syrup at levels >10%, achieving overall (authentic plus adulterated) correct classification rates of >92.2 using k-nearest neighbors. Correct classification rates of 82.4% were also obtained for adulterantion of the apple juice with a synthetic solution of fructose, glucose, and sucrose (*53*). A large trough at 1064 cm^{-1} and a peak maximum at 1033 cm^{-1} monitored the fructose/glucose ratio. Quantitative analysis using a PLS regression procedure effectively quantified adulteration produceding a root-mean-square error of cross-validation (RMSECV) value of 4.9% with a correlation coefficient equal to 0.89, showing promise as a rapid screening technique for the detection of a broad range of potential adulterants in apple juice (*31*). Differentiation of commercial orange juice products by FT-MIR has shown similar class separation compared to the data generated from an electronic nose (e-nose), headspace gas chromatograpy, a mass spectrometer-based chemical sensor, and physical/chemical measurements (*54*). The screening capabilities of FT-MIR spectroscopy have been evidenced by limited presence of false positive results (Table 2). In general, the greater sample

variability incorporated into the pattern recognition analysis, produces models of higher accuracy (*31*).

He and collaborators (*15*) showed the clustering of commercial juices using attenuated total reflectance infrared spectroscopy and soft independent modeling of class analogy (SIMCA). The application of solid-phase extraction-enriched phenol compounds provided signature-like spectral information that allowed for the differentiation of juices with different origins and zero percent misclassification (Figure 4). It was concluded that the infrared technique, assisted by a simple fractionation and chemometrics, provided a promising analytical method for the assurance of juice quality and authenticity.

By combining the MIR spectral data with SIMCA statistical analysis, a very high level of discrimination was achieved based on type of grape (*15*). Grape juice samples were obtained from multiple lots and growing locations showing that spectral differences were detected between different varieties of grape juice, allowing for effective discrimination (Figure 5). This demonstrates the model's suitability for varietal discrimination as well as the high level of reproducibility in the method.

One metric by which to gauge the discriminating power of a SIMCA model is interclass distance (ICD). The interclass distances assign a numeric value to represent the distance between two SIMCA classes in the principal component space. Therefore, the larger the interclass distance between two classes, the higher the level of discrimination the model has achieved between the samples in those classes. In the above example, the interclass distances ranged from 17 to 41, generally an interclass distance of 3 is considered well discriminated.

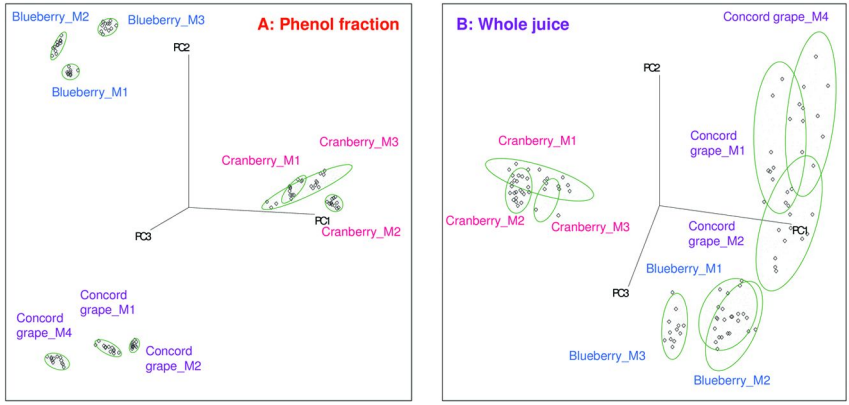

Figure 4. SIMCA class projections plots for the whole juice (A) and the phenol-rich fraction (B) spectral data (15).

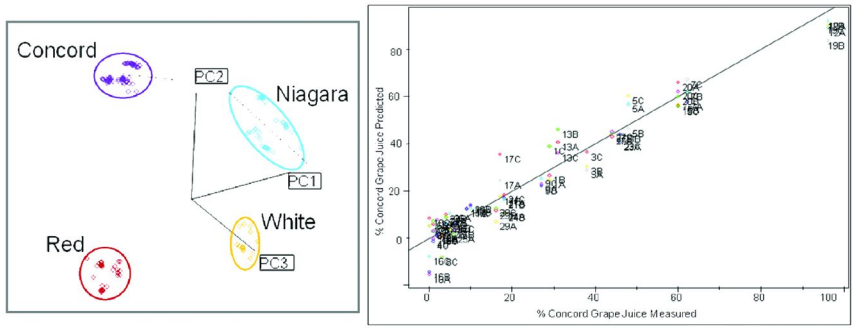

Figure 5. A) SIMCA 3D Class projections of 100% grape juices from 4 different varieties and 14 different lots. B) Y-Fit of PLS of 4 grape juice blends.

A simulated annealing algorithm was then used to create a small representative sample set from the four different juices and 17 different lots to develop a calibration data set that was used to supervise the building of a PLS model. Considering the wide amount of variability, this model was capable of explaining a very large amount of the variance between the FT-IR spectra associated with different grape juice blends (*55*, *56*). The Y-Fit shows a good linear fit meaning that the model's internal validation methods are making good predictions about grape juice content based on the FT-IR spectral data (Figure 5B). The PLS model explained 99% of the variance in the data using 10 factors giving a standard error of validation (SECV) of 2.63%. This model was validated against a separate set of samples and was shown to have an error of prediction of ±8.4%. Thus, FT-IR coupled with chemometrics is a feasible method for the discrimination of pure grape juices on the basis of variety and for the prediction of Concord grape juice in grape juice blends.

Summary

The juice and wine industries are increasingly concerned with adulteration in terms of lower grade ingredients which can affect not only the quality of the finished product but represent economic fraud and may have health implications to consumers. These factors have underlined the need for rapid, reliable, easy-to-use and cost-effective techniques that can effectively authenticate the purity of fruit juices for the juice industry and regulatory agencies.

Improved design and auxiliary optics in Fourier transform infrared (FT-NIR and FT-mid-IR) spectroscopic instrumentation combined with multivariate data analysis allow monitoring changes of the entire chemical composition in the food matrix based on its molecular "fingerprint". Adulteration or any variation attributed to variety or source difference might be elucidated via pattern recognition methods.

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These analytical techniques allow for nearly real-time measurements, simple data acquisition and immediate predictions with minimal personnel training, and cost. Furthermore, simple fractionation steps can improve the differentiation power notably by separating fruit juice into polar (sugar and simple acids rich) and non-polar (phenols rich) fractions.

Miniaturization of optical sensors has facilitated the translation of benchtop-style spectroscopy into portable and handheld systems that could be effectively deployed in challenging environments by nontraditional users. Yet, to become a routine screening tool, additional research is needed to improve sample preparation and method sensitivity, to generate database libraries and to evaluate powerful multivariate classification methods. The ability to rapidly and efficiently screen large number of samples, while detecting differences, even when the source of the differences is unknown makes IR an ideal tool for protection of food from intentional or unintentional adulteration.

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Chapter 21

Quality and Authenticity Control of Fruit-Derived Products

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The authentication of fruit products such as fruit preparations and jams is most challenging because of their complex composition. In particular the determination of the fruit content, which is an important quality trait of fruit-derived products, is difficult. The situation is further aggravated by the lack of reliable methods. Previous approaches targeted at the determination of low molecular compounds were of limited usefulness because these can easily be manipulated. In contrast, hemicellulose, a high molecular constituent of the plant cell wall, proved to be a promising parameter for the quantification of the fruit content and even for fruit species determination. This contribution provides an overview of our recent investigations on quality and authenticity control of fruit preparations and jams, with a focus on fruit products made from strawberries, cherries, apricots, and peaches.

The adulteration of foods represents a serious economic issue. Fraudulent practices are not an invention of our time but have been observed throughout the history of food production (*1*), especially since profit margins in the food sector are comparatively low. Adulteration may occur in very different forms and affect virtually all food commodities including functional foods and natural health

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products. For example, milk and dairy products have always been a "popular" target, whether through watering or the addition of melamine. The detection of such types of adulteration is relatively straightforward.

In contrast, the detection of fraudulent manipulations of fruit products has proved to be challenging in the past, in particular in the case of fruit preparations and jams. Fruit preparations are important intermediates used for the production of dairy products, e.g. fruit yogurts, and in bakery products and ice cream. While there is no food law in place in Germany that governs the composition of fruit preparations, manufacturers are obliged to meet the requirements established by the German Federation of Food Law and Food Science (BLL) for fruit preparations. These guidelines stipulate a fruit content of 35% for fruit preparations in general, whereas preparations based on raspberry, raspberry/blackberry, black and red currant, gooseberry, banana, and pineapple require a fruit content of 25-30%. According to the BLL guideline, the fruit content of yogurt products shall be between 1.5% and 6%, depending on the type of yogurt. The fruit content of 'fruit yogurt' needs to be 6%, whereas 'yogurt with fruit preparation' contains only 3.5% fruit. The fruit content may even be lower (<3.5%) in 'yogurt with fruit taste'. Thus, it is evident that the fruit content is an important quality trait of fruit preparations.

Fruit preparations are highly complex products which contain one or more species of fruit, hydrocolloids, sugars, essences and flavors, acidulants, and coloring foodstuffs, if necessary. The fruit, or fruit constituents, shall be healthy and fresh, unfermented, and in a state of maturity that is suitable for processing. Juices added for coloring purposes are not regarded as "fruit". As a result of their complex composition, quality and authenticity control of fruit preparations, in particular the determination of the fruit content is extremely difficult. Common types of adulteration are the reduction of the fruit content and the addition of cheaper fruits and bulk materials to feign a higher fruit content, both leading to an inferior product quality.

Previous approaches targeted index compounds like dry matter, ash, insoluble solids, sugar profile, amino acids, and phenolic compounds. Even the number of achenes in strawberry jams was taken as an indicator of their fruit content. However, because these constituents can easily be manipulated and are subject to considerable variations, they cannot be considered reliable parameters for the determination of the fruit content. As shown for tomato ketchup, PCR techniques are limited in the authentication of heated fruit products since the DNA is degraded under acidic conditions (*2*). For a comprehensive treatise of the methods used for quality and authenticity control of fruit purées, fruit preparations, and jams we refer to a review published by Fügel et al. (*3*).

In previous investigations we demonstrated that cold storage and calcium brining significantly increase the firmness of canned strawberries and cherries, and provided evidence that the composition of pectins is a crucial factor in the textural improvement of fruits (*4*). These studies have also revealed a remarkable stability of the hemicellulose (HC) fraction during processing. Therefore, we hypothesized that HC could be a suitable parameter for the determination of the fruit content of fruit products and developed analytical protocols for the isolation of HC from

fruits, fruit preparations, and jams. This contribution is intended to summarize the results of our investigations on quality and authenticity control of fruit products.

Fractionation and Characterization of Cell Wall Polysaccharides

We investigated the cell wall polysaccharide composition of economically important cultivars of strawberries, cherries, apricots, and peaches. In addition, 2 apple cultivars were included to obtain preliminary data on their profile of neutral sugars. Also, 4 pumpkin cultivars were assessed because of their potential use in fruit products based on yellow fruits to improve the visual appearance and feign a higher fruit content.

The isolation of the HC fraction requires first the recovery of the alcohol insoluble residue (AIR), which was accomplished by homogenizing the lyophilized fruits in boiling ethanol. Subsequently, the AIR was partitioned into water-, oxalate-, acid-, and alkali-soluble pectins, HC, and cellulose fractions. The HC fraction was hydrolyzed and the profile of monosaccharides determined by capillary electrophoresis (*5*) or gas chromatography (*6*) after derivatization of the sugars.

Striking differences were found for the neutral sugar profile of the HC fractions of the fruit species investigated. The HC fraction of strawberries was characterized by relatively low amounts of arabinose (4.6-6.2% in the AIR) and higher levels of xylose (18.3-22.3%) and galactose (10.8-13.7%). The glucose content was >40% in all strawberry cultivars. The HC fraction of cherries showed 15.1-18.3% arabinose and 20.1-20.8% galactose. The glucose content ranged between 31% and 33% and was lower than in strawberries. The neutral sugar profile of the HC fractions of apples and strawberries was found to be very similar. As a result, fraudulent admixtures of apple purées to strawberry fruit preparations would not be detectable using this method, whereas blends of apple and cherry products would show decreased arabinose contents (*5*).

Astonishingly, the neutral sugar composition of the HC fractions of yellow fruits also revealed significant differences although peaches and apricots belong to the same genus (*Prunus*). Apricots contained 9.8-12.2% mannose and 4.1-7.2% arabinose, whereas in peaches lower contents (3.9-6.7%) of mannose but higher contents (11.4-17.3%) of arabinose were found. Pumpkins were characterized by somewhat lower levels of galactose (6.5-12.2%) compared to contents of around 16% in apricots and peaches. Mannose was found only in trace amounts (*6*).

The HC content in the AIR proved to be rather constant in the fruits. In strawberries, 12.7-14.6% was found, whereas in cherries the HC content ranged from 15.1% to 16.7% (*4*). HC levels in apricots were between 9.7% and 12.2%. In peaches we observed HC contents from 11.8% to 15.9%. Only one peach cultivar showed higher contents of 17.9% (*6*).

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Determination of the Fruit Content of Fruit Products and Dairy Products

Because of the striking constancy of the levels of the HC fraction and its exceptional stability during processing we decided to use this cell wall fraction as an analytical tool for the determination of the fruit content of fruit products, in particular fruit preparations. However, we first needed to establish a correlation between the fresh weight of fruits and the HC fraction. For this purpose, we defined the 'conversion factor', which allowed us to calculate the fruit content after determining the amount of HC in the fruit products (*7*). After recovery of the AIR from the fruits and fruit products, respectively, the HC fraction was isolated as described above by sequential extraction and dialysis. Strawberry fruit preparations with fruit contents ranging from 30% to 60% were produced to assess the suitability of the method. An excellent agreement of the specified and determined fruit contents was obtained in most cases, with absolute deviations from the initial fruit contents usually not exceeding 2.4%. Only for the sample with the highest fruit content (60%) we observed an overestimation of approximately 4% (*7*). Also in the case of cherry fruit preparations excellent results were obtained when single hydrocolloid systems and less complex combinations of hydrocolloids were used (26.8% vs. 30%; 38.6% vs. 40%; 42.5% vs. 40%; 37.6% vs. 40%; 41.2% vs. 40%). However, the deviations were larger (46.2% vs. 40% and 49.6% vs. 40%) when fruit preparations were produced using 3 hydrocolloids for stabilization (*8*). The latter required the enzymatic digestion of the added matrix compounds.

In continuation of the above studies on red fruits, we expanded our investigations to self-made and commercial apricot and peach fruit preparations. Furthermore, jams and spreads made from apricot and strawberry fruits were included. The calculated fruit contents of most self-made fruit products (fruit preparations, jams) were in good agreement with the specified contents, with deviations ranging from 0.3% to 4.1%. Only in one case a considerable overestimation of 6.5% was observed. Remarkably, the determined fruit content of all commercial fruit preparations, jams, and spreads was well in agreement with the specifications obtained from the producers (*9*). These results are of particular interest because the detailed compositions of the fruit products as well as the cultivars of the fruits used for the manufacture of the jams, spreads and fruit preparations were not known.

Since fruit preparations are often used as ingredients in fruit yogurts, the method was applied also to the determination of the fruit content of strawberry yogurt. For this purpose, individually quick frozen strawberries were blended with a commercial yogurt in proportions of 6, 10, 20, and 30%. Subsequently, the blends were homogenized. After digestion of the protein matrix using proteases and lyophilization of the sample, the AIR was prepared and extracted sequentially to isolate the HC fraction. While satisfactory results were obtained for the yogurt sample with high fruit content (30% vs. 31.5%), considerable overestimations were observed in cases of fruit contents, probably caused by the incomplete extraction of high molecular compounds (*10*).

Conclusions

The results obtained from the above mentioned studies demonstrate that the HC fraction is a promising parameter both for the determination of the fruit content of fruit-derived products and for the authentication of fruit species. The absolute amount of the HC fraction provides information about the fruit content, whereas the neutral sugar profile can be used, although with some limitations, for the differentiation of fruits. Most importantly, this method does not require information about the composition of the fruit products because all ingredients typically added during manufacture are removed through extraction and enzymatic digestion. However, although only simple analytical methods were used, that is, gravimetry, gas chromatography, and capillary electrophoresis, it should be noted that the approach presented is not a rapid method. In particular, sample preparation including lyophilization, sequential extraction, and dialysis take several days. As a result, a high sample throughput cannot be accomplished. Therefore, we recently developed an analytical procedure that employs Fourier transform near infrared spectroscopy and chemometrics for the rapid determination of fruit authenticity and for the quantification of the fruit content (*11*).

Fraudulent practices will continue also in the future and most probably one single method will not be sufficient to detect all possible types of adulterations of fruit products. However, we feel that the availability of as many methods as possible will increase the expenditures of perpetrators to an extent that finally makes adulterations uneconomical. Therefore, efforts targeted at quality and authenticity control of fruit-derived products should be continued also in the future.

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Chapter 22

Authentication of Saffron Spice (*Crocus sativus* L.)

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Saffron spice is highly valued both in cookery and in the food industry due to its coloring properties, alluring aroma and pleasant bitter taste. It is also highly appreciated in the cosmetic and pharmaceutical industry for its biological and medicinal virtues. The authenticity of saffron is an important matter in view of consumer protection, quality assurance, active properties and economic impact. This spice was and still remains the most expensive in the world. Consequently, saffron has undergone a wide range of adulterations and authentication is a permanent challenge. This chapter commences with a revision of the main characteristics of genuine saffron and goes on to deal with the international standards and legislation used to certify saffron; the history of saffron adulteration and the most known fraudulent practices; and the methods used for the assessment of authenticity and detection of adulterants. Finally, the perspectives concerning the authentication of this product are discussed.

Introduction

Saffron spice consists of the dried stigmas of *Crocus sativus* L. blossoms. Each flower has one stigma, divided into three red filaments, at the tip of a yellow-white style portion. This spice may be presented in filaments, in cut filaments or in powder form, which is more prone to adulteration. Its coloring, tasting and flavoring properties, all rolled into one, make saffron highly valued both in

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cookery and in the food industry. Its biological activities and medicinal virtues are also highly appreciated in the cosmetic and pharmaceutical industry (*1*). The authenticity of saffron is an extremely important matter for these industries and for consumers in view of security and protection, quality assurance, active properties and, last but not least, economic impact.

Despite the fluctuation in saffron prices on international markets, this spice was and still remains the most expensive in the world due mainly to its extremely laborious manual harvesting and processing. Consequently, a wide range of adulterants and substitutes has appeared throughout its history. These adulterants or substitutes rarely have the active properties of saffron and may even be harmful for health. Therefore, authentication has been a permanent challenge.

From the eastern Mediterranean region where this crop is believed to have originated, saffron cultivation spread first to Europe and Asia and then to the other continents. In the last years, annual world production is about 200 tons and the main producer is Iran (which accounts for approximately 90% of total production), followed by India (5%), Greece (2%), Spain (1%) and Morocco (less than 1%) (*2*). As regards the world saffron market, according to the United Nations Comtrade Database (*3*) in 2005 Iran followed by Spain were the principal exporters. As regards imports, Spain, Italy and the USA stand out in this order. The year 2005 was selected because data from Iran were still included. As previously mentioned, Iran is currently the main producer although Spain, the former main source of saffron spice, is at the forefront of world trade with approximately one fourth of the total exports. Besides its local production, Spain, which has traditionally produced the highest quality saffron known as *La Mancha* saffron, is devoted to processing the raw material from other countries and exporting the processed product. This dynamic trade enables saffron to take advantage of the experience and know-how of some countries to improve saffron quality, but also makes strict authenticity tests necessary.

Saffron samples from different geographical locations and from different processing methods show considerable variation in quality. This is due to the influence of edaphoclimatic crop conditions, harvesting, stigma separation, handling, storing, packaging and, above all, the drying process (*4–6*). For this reason and for the differences in cost production, the price of wholesale saffron from various geographical areas differs greatly. In the last decades, there has been a growing concern for guaranteeing and defending the quality of saffron historically produced in certain regions (*7*) and many Protected Designations of Origin (PDO) and quality labels have been created in Europe: *Azafrán de La Mancha, Artesanía Alimentaria de Aragón, Munder Saffran, Zafferano di Sardegna, Zafferano purisimo de San Gimignano, Zafferano delle Colline Fiorentine, Zafferano dell'Aquila* and *Krokos Kozanis*. In consequence, authentication of saffron origin is also an important issue.

Recent advances in saffron chemical characterization, the application of new analytical and chemometric techniques, together with the study of a large number of samples from all over the world, have contributed to the advances in saffron authentication summarized in this chapter.

Characteristics of Genuine Saffron

According to saffron spice definition, genuine saffron should have the morphological, physical and chemical characteristics of the dried stigmas of *Crocus sativus* L. These characteristics confer to saffron its outstanding color, taste and aroma as well as its valued coloring, tasting and flavoring properties.

Color and Coloring Properties

Color is the most important quality characteristic and coloring strength values ($E^{1\%}_{1cm}$440 nm) of its aqueous extracts are critical for the commercial value of the spice. The red color of saffron spice and its coloring capacity to give yellowish-red hues are due to crocetin esters, a group of water-soluble carotenoids that derive from crocetin ($C_{20}H_{24}O_4$, 8,8′-diapo-Ψ,Ψ′-carotenedioic acid) where glucose, gentiobiose, neapolitanose or triglucose are the sugar moieties and where *trans*- or *cis*-configuration is found. These crocetin esters are also known as crocins. The structure and spectral characteristics of saffron pigments have been thoroughly researched. Carmona et al. (*2*) reviewed the literature on crocetin esters chemical characterization, analysis, degradation and biosynthesis. In addition, the authors reported new studies on the influence of the dehydration process on crocetin ester content, the relationship between visual color and coloring strength, the evolution of crocetin esters during accelerated aging, and their aroma generation. This group of compounds represents between 0.5 and 32.4% on a dry basis (*8*). Both the presence and proportion of each crocetin ester are characteristic of saffron and they are influenced by the production process. The heterogeneity of saffron (from different origins, submitted to different dehydration processes and of different qualities) together with the lack of commercial standards of sufficient purity for each crocetin ester and the various methods of analysis explain the wide range of crocetin ester content reported in the literature. Another source of crocetin esters and thus, a potential substitute or adulterant of saffron, is gardenia fruit (*Gardenia jasminoides* Ellis) from which a natural yellow colorant also known as gardenia and used in food and oriental medicine is obtained.

Taste and Tasting Properties

Together with its alluring aroma, its pleasant bitter taste is what mainly differentiates saffron from other natural or synthetic colorants such as safflower (*Carthamus tinctorius* L.), turmeric (*Curcuma longa* L.), gardenia, and tartrazine. Picrocrocin (4-(β-D-glucopyranosyloxy)-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde) is considered to be the foremost contributor to saffron's bitter taste, although other compounds such as picrocrocin-related ones, kaempferols and amino acids with this organoleptic property have been characterized in saffron spice. Contents of picrocrocin in saffron spice from 0.8 to 26.6% on a dry basis have been reported (*8–10*). It is known that picrocrocin is converted to safranal (the major compound in the saffron volatile fraction contributing to its aroma) either by a two-step enzymatic/dehydration process involving the intermediate 4-hydroxy-2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde

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(HTCC) or directly by thermal degradation. There is also evidence of this conversion at extreme pH (*11*). Only a few studies deal with the taste of saffron and, in particular, with picrocrocin and bitter taste (*12*, *13*). In this last work, the taste detection threshold of picrocrocin was set at 10 mg L^{-1}. To our knowledge, picrocrocin sources are limited and neither potential substitutes nor adulterants of saffron contain it. Therefore, picrocrocin can be the biomarker in saffron authentication. The flavonoid fraction of saffron is made up of glycosides of kaempferol, among which the presence of the kaempferol-3-*O*-sophoroside-7-*O*-glucoside, the kaempferol-7-*O*-sophoroside, the kaempferol-3-*O*-sophoroside, the kaempferol-3,7,4′-triglucoside, and a kaempferol tetrahexoside have been reported (*14*, *15*). A recent study of free amino acids revealed that alanine, proline and aspartic acid are the major amino acids in saffron spice (*16*).

Aroma and Flavoring Properties

The compounds that make up saffron aroma and its glycosidic precursors have received much attention in recent years (*14*, *17*). More than 160 volatile compounds have been reported in this spice, though some authors asserted that many of them could be generated as artefacts when exhaustive isolation procedures are employed (*17*). Safranal (2,6,6-trimethyl-1,3-cyclohexadiene-1-carboxaldehyde) is the major compound in the volatile fraction of saffron, representing around 70% and between 1.07 and 5.670/000 of dry matter weight (*18*). Other main compounds in terms of aroma chemistry are 3,5,5-trimethyl-2-cyclohexene-1-one (isophorone), 2,6,6-trimethyl-2-cyclohexene-1,4-dione (4-ketoisophorone), 3,5,5-trimethyl-3-cyclohexen-1-one (an isomer of isophorone), 2,6,6-trimethyl-1,4-cyclohexadiene-1-carboxaldehyde (an isomer of safranal), 2,2,6-trimethyl-1,4-cyclohexanedione, HTCC and 2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one. High quality saffron presents aroma characteristics such as sweet, floral, and spicy. However, there are peculiarities in the samples coming from the different countries where saffron is produced traditionally. In fact, dealers are capable of recognizing, from its aroma, the origin of the most characteristic, significant saffron as far as production quota and presence on the market is concerned. For example, the content of 2-phenylethanol in saffron varies according to the spice's preparation and is related to the content of pollen from the stamens. The preparation process used in Spain makes this content one of the lowest since stigmas are stripped from the rest of the flower by hand and during dehydration, stigmas are almost completely stamen-free. In other producing regions, where stigmas and part of the stamens are dried together and separated later, the pollen content is much higher and aromas can be transmitted from contact during dehydration. The presence of pollen does not suppose a problem if it does not surpass limits established in quality control standards, and could be taken into consideration when according genuineness to saffron from a determined production origin in order to avoid fraudulent labeling.

Active Properties

Saffron, its extracts and tinctures have been used for medicinal purposes in many cultures since ancient times. In general, saffron is used in folk medicine as an antispasmodic, eupeptic, gingival sedative, anti-catarrhal, nerve sedative, carminative, diaphoteric, expectorant, stimulant, stomachic, aphrodisiac, anti-inflammatory and emmenagogue remedy. Apart from these properties, Ríos et al. (*19*) pointed out three potential therapeutic applications of saffron active constituents: antitumoral, hypolipidaemic and tissue oxygenation enhancement. Modern pharmacological research and incipient clinical testing largely confirm traditional knowledge regarding the medicinal effects of saffron. Schmidt et al. (*20*), in a review of the preclinical and clinical literature, highlighted two major indications for saffron extracts, namely cancer and minor depression. In fact, pharmacological research currently focuses on antitumoural effects, whereas clinical testing has provided evidence for the traditional well-known antidepressant effects. Another discovery commented by these authors is a significant improvement in learning abilities and memory. There is no doubt that authenticity and a detailed quality assurance of the saffron is a prerequisite for using this product for its medicinal virtues and in preclinical and clinical trials.

International Standards and Legislation Used To Certify Saffron

Saffron quality in international trade has been mainly determined by specifications recommended by the ISO 3632 standard. It was first published in 1975 and has undergone three revisions: in 1980, 1993 and 2003. The latest revision has given rise to Technical Specification ISO/TS 3632 (*21*) which is still under discussion in Technical Committee ISO/TC 34, Subcommittee SC 7. After all these revisions, ISO/TS 3632 is very complete and, although it is not compulsory, at present this is the internationally accepted reference specification for saffron quality control and it is used in most transactions. The strict requirements resulted in the disappearance of very low quality saffron and have made the detection of adulterations much easier. According to ISO/TS 3632-1 (*21*) saffron is considered to be pure when it complies with the requirements in Table I and when no matter has been added to the natural product. As also shown in Table I, at the international level, ISO/TS 3632 classifies saffron into three categories with regard to the specified physical and chemical parameters, although it does not establish the content of crocetin esters, picrocrocin and/or safranal. Among these categories, category I corresponds to the highest quality saffron.

Also, at a national level, pure saffron is graded according to its characteristics giving rise to different commercial categories. In Spain examples of this classification (from the best to the lowest category) are the categories "*Selecto or superior*", "*Río*", "*Sierra*", "*Common or Standard*", "*Coupe*" and "*Ground*" (*22*); in India: "*Lacha*" and "*Mongra*" or in Iran: "*Sargol*" and "*Pushali*". Table I also shows the specifications that all saffron under the *Azafrán de La Mancha* PDO (*23*) should meet. Besides the geographical area from which the saffron comes, all details are compiled on how to obtain, process, package or store it. In order to

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make authentication easier, saffron from this PDO is presented to the consumer in filaments and never powdered. Only saffron from the latest harvest obtained from registered fields can be packaged under this label, in containers with a maximum net content of 100 g. There is a traceability system from the field to the final product to guarantee its authenticity.

Fraudulent Practices

Pliny the Elder said, "Nothing is adulterated as much as saffron" in the 1st century. Its scarcity and high value have made saffron spice susceptible to adulterations with a great variety of vegetal, animal or mineral materials since ancient times. In times of Marco Polo (13th century), for example, saffron reached higher prices than gold.

Adulterants and substitutes mainly consist of vegetal parts of the plant of *Crocus sativus* or from other plants like marigold (*Calendula officinalis* L.) or arnica (*Arnica Montana* L.), which might have been dyed. Many diverse plant materials use the name "saffron" all over the world, e.g. safflower (*Carthamus tinctorius* L.) is called bastard saffron or saffron thistle; marigold is also known as Indian saffron, American saffron or Mexican saffron. These names contribute to the misidentification of saffron in filaments. Also turmeric (*Curcuma longa* L.) can be misidentified as powered saffron. The mixture with extracted (recolored exhausted saffron) or old saffron also constitutes a fraudulent practice. In addition, the increase in moisture and the addition of substances like honey, starch, meat fibers, colored artificial fibers or even inorganic compounds to increase weight constitute known adulterations (for an overview cf. refs. (*24–26*)).

Nowadays, addition of artificial colorants is the most common way of fraud. The aim of this practice is to mislead the consumer by improving or changing the appearance of old and low quality saffron or of other extraneous materials added in order to increase weight or used as substitutes. Moreover, gardenia has been found among the latest adulterants and substitutes of saffron due to its content in crocetin esters.

Misbranding or falsification of origin may also be a lucrative activity. For example, before 1999 when the designation "*Azafrán de La Mancha"* was protected, the word "*Mancha*" designated the current commercial quality "*Selecto or Superior*". It was legal to import and export saffron from any origin under the label "*Mancha*" provided that it fulfilled the requirements established by the External Trade Normatives at that moment. Taking advantage of legislative vagueness and the excellent reputation and higher price of saffron historically produced in La Mancha, some dealers packaged good quality saffron that had not been produced in Spain under this denomination. Currently, "*Mancha*" can only be used to designate saffron produced in a specific geographic area of Castilla-La Mancha.

Table I. Requirements of saffron according to ISO/TS 3632-1 (*21*) and characteristics of saffron produced under the *Azafrán de La Mancha* PDO (*23*)

Characteristics	*ISO/TS 3632-1 (2003) Specifications*			*"Azafrán de La Mancha" Specifications*	
	Category I	*Category II*	*Category III*	*Bulk saffron*	*Packaged saffron*
Floral waste, maximum %	0.5	3	5	0.5	0.5
Foreign matter, maximum %	0.1	0.5	1.0	0.1	0.1
Water and volatile matter, maximum % Saffron in filaments Powdered saffron	 12 10	 12 10	 12 10	 7–9	 11
Total ash, as dry matter, maximum %	8	8	8	—	8
Acid-insoluble ash, %, as dry matter, max.	1.0	1.0	1.5	—	1
Soluble extract in cold water, as dry matter, %, max.	65	65	65	—	65
$E^{1\%}_{1cm}$ 257 nm on dry basis, min.	70	55	40	70	70
$E^{1\%}_{1cm}$ 330 nm Minimum Maximum	 20 50	 20 50	 20 50	20	20
Coloring strength, $E^{1\%}_{1cm}$ 440 nm on dry basis, min.	190	150	100	200	200

Continued on next page.

Table I. (Continued). Requirements of saffron according to ISO/TS 3632-1 (*21*) and characteristics of saffron produced under the *Azafrán de La Mancha* PDO (*23*)

Characteristics	*ISO/TS 3632-1 (2003) Specifications*			*"Azafrán de La Mancha" Specifications*	
	Category I	*Category II*	*Category III*	*Bulk saffron*	*Packaged saffron*
Artificial water-soluble acid colorants	Absent	Absent	Absent	Absent	Absent
Safranal content (% of total volatile components), min.				65	65
Ether extract, % (w/w)				—	3.5−14.5

Laws to protect the purity of saffron have been imposed throughout history to pursue fraudulent conduct. In the 15th century, an armed police force was created in Italy called "Uficio dello Zaferano". Established in Venice, its mission was to ensure that the spice would not be adulterated. In France, Henry II (1519-1559) established confiscation, burning of the merchandise and corporal punishment for the adulterators (*27*). Henry VIII (1509-1547), fanatically devoted to its aroma, forbad the use of saffron as an article for dyeing clothing and hair and any act of falsification at that time received the death penalty. In the Middle Ages, there were also saffron inspectors in Nuremberg, Germany, with authority to burn or bury alive those who adulterated saffron (*28*).

Nowadays, national and international standards to control saffron purity must be observed. If not, the commodity is usually withdrawn and important economic fines are given.

Methods for Saffron Authentication

Some of the adulterations can be detected easily by rather simple methods. First we will mention the immersion of saffron in water, because saffron immediately expands into a characteristic form that is easily distinguishable from Crocus stamens or florets of safflower, marigold or arnica. Secondly, its dyeing properties can be used to authenticate saffron since a 1% aqueous solution in contact with cotton, silk or wool for 30 minutes stains the first one orange, the second yellow and leaves the third uncolored. Safflower, calendula leaves, logwood, sandalwood, Brazilwood or turmeric do not turn cotton orange (*25*). For identification test, ISO/TS 3632 (*21*) proposes the examination of saffron in filaments and cut filaments with a magnifying glass and the use of colored reactions for powdered saffron. Pure saffron immediately turns to a blue color which rapidly turns reddish brown when a diphenylamine and sulfuric acid solution is added. In the presence of nitrates, the blue color persists. A wide range of tests based on color reactions was summarized by Sampathu et al. (*25*). ISO/TS 3632 (*21*) also proposes microscopic examination in order to determine whether the sample consists exclusively of stigmas belonging to *Crocus sativus* L. and to highlight any floral waste and foreign matter.

The extensive knowledge of the chemical composition of saffron and the different spectrometric and chromatographic methods for the analysis of its constituents have generated the fingerprints of the spice that enable its authenticity control.

Ultraviolet-Visible (UV-vis) Spectrophotometry

The peculiarities of the raw material and characteristics of companies that process and package saffron spice (seasonal production, batches of even less than 1 kg, small and medium-sized companies) make the UV-vis spectrophotometry essential in routine quality control. Despite the high price of this spice, most companies of this sector cannot afford more expensive analytical techniques such as high performance liquid chromatography (HPLC) or gas chromatography (GC).

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The typical UV-vis spectrum of a saffron aqueous extract is shown in Figure 1A. It has three absorption bands with maxima at about 257, 330, and 440 nm. In pure methanol saffron showed four peaks at 253, 319, 430, and 455 nm (λmax = 430 nm), while methanol-water (50:50) resulted in three peaks at 256, 323, and 437 nm (λmax = 437 nm) (*29*).

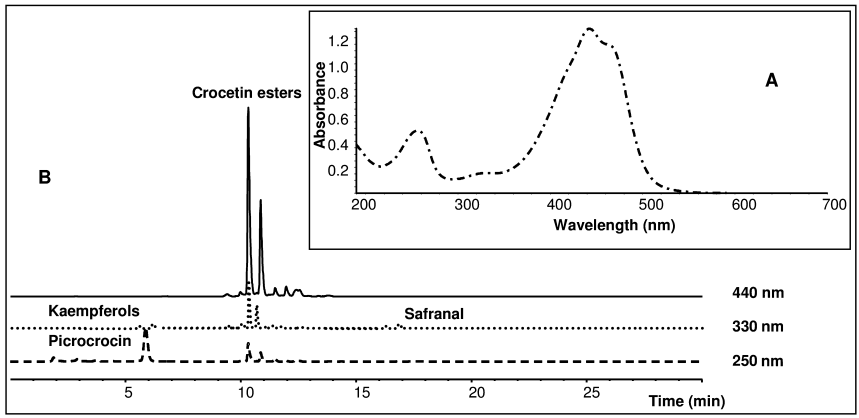

Figure 1. (A) UV-vis spectrum of saffron spice, 50 mg/L in water; (B) HPLC chromatograms at 250, 330 and 440 nm of saffron extract.

Moreover, the use of the parameters $E^{1\%}_{1cm}$ at 257 nm, $E^{1\%}_{1cm}$ at 330 nm, $E^{1\%}_{1cm}$ at 440 nm (coloring strength) to classify saffron according to ISO/TS 3632 (*21*) has led to the existence of a spectrophotometer in almost all saffron companies. These parameters take into account that picrocrocin, safranal and crocetin esters also have a maximum at these wavelengths in their UV-vis spectra. The coloring strength is representative of the total crocetin ester content, although this procedure by itself does not allow to distinguish their detailed composition, that is, each *trans*-crocetin ester and each *cis*-crocetin ester. However, the content of safranal and picrocrocin through the parameters $E^{1\%}_{1cm}$ 330 nm and $E^{1\%}_{1cm}$ 257 nm of the aqueous extract, respectively, cannot be determined due to the low solubility of safranal in water and interferences of other compounds of saffron extract, primarily crocetin esters (*10*, *29–31*). Del Campo et al. (*32*) studied 345 saffron samples and reported an overestimation of the picrocrocin content obtained through the values of $E^{1\%}_{1cm}$ 257 nm due to these interferences, especially in those samples with a low trans/cis crocetin ester ratio. The range of $E^{1\%}_{1cm}$ 257 nm found in the large amount and variability of samples analyzed, the authors proposed an increment up to 50 units in the minimum value for category III

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and up to 60 units for category II in ISO/TS 3632 (Table I). Furthermore, these limitations have prompted new research on the application of multivariate models able to determine the content of the main crocetin esters and picrocrocin from spectrophotometric data (*10*) and the application of solid phase extraction as a previous step preventing interferences in the determination of picrocrocin by UV-vis spectrophotometry (*33*). Also the use of non polar extraction of safranal before measuring with this technique has been proposed to the ISO committee in charge of ISO/TS 3632 revision (*34*).

It was not until the last revision of ISO 3632 that the UV-vis fingerprint of saffron aqueous extracts was included. Several works have demonstrated its ability to detect certain adulterations with colorants either to increase its coloring strength or to improve appearance. Carmona et al. (*35*) reported the changes occurring in the UV-vis spectra between 190 and 700 nm when saffron is adulterated with 14 water soluble colorants (Table II) in order to increase the coloring strength 1, 5 and 10 units and highlighted the capacity of this technique to detect 12 of them. In the European project SAFFIC (*34*) the effect of adding different colorants on the UV-vis spectrum of saffron in order to detect adulterations were also studied (Table II). Moreover, second derivative spectra of saffron aqueous extracts and multivariate calibration after sample pretreatment have proven useful as screening methods for the detection of artificial colorants (*36*, *37*). As erythrosine is pH dependent and could not be detected under the above conditions, fluorescence detection has been studied (*38*).

Fourier Transform-Infrared (FT-IR) and FT-Raman Spectroscopy

Tarantilis et al. (*39*) studied the FT-IR and FT-Raman spectra of crocetin esters, dimethylcrocetin and crocetin. The FT-IR spectra of these compounds have characteristic absorbance bands between 1706 and 1664 cm^{-1} ($\nu_{C=O}$) and in the region between 1243 and 1228 cm^{-1} (ν_{C-O}). Two main Raman lines were observed near 1540 and 1166 cm^{-1} which were assigned to ($\nu_{C=C}$) and (ν_{C-C}) stretching modes, respectively. The FT-IR and FT-Raman spectra revealed very complementary structural information for these compounds. In addition, the authors claimed that FT-Raman could be used to study the molecular interactions of saffron carotenoids in vitro and in living cells and for the non-destructive analysis of natural products.

Assimiadis et al. (*40*) also applied FT-Raman spectroscopy for qualitative measurements of carotenoids in saffron and Aminzadeh (*41*) proposed the quickly attainable FT-Raman spectrum of solid saffron for saffron standardization. However, the feasibility of this technique for a quantitative approach has not been studied until recently (*42*). Anastasaki et al. have reported the feasibility of Raman spectroscopy in combination with partial least-squares (PLS) regression for predicting the content of crocetin esters and coloring strength. Compared to liquid chromatography and UV-vis spectrophotometry, Raman spectroscopy gave accurate data and was proposed as a tool for rapid screening of quality in saffron samples (*42*).

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Fourier transform-Near-Infrared spectroscopy (FT-NIR) has been studied for determination of saffron chemical composition and geographical differentiation (*32*, *43*). The results showed the potential of this technique when applied to saffron quality control, obtaining results quickly and with minimum sample manipulation. Calibration and validation procedures with the results obtained by UV-vis and HPLC with diode-array detection (HPLC-DAD) measurements demonstrated its capacity to determine moisture and volatile content, coloring strength, $E^{1\%}_{1cm}$ 250 nm, and $E^{1\%}_{1cm}$ 330 nm and the content of the five main crocetin esters and picrocrocin.

Comparing the results of this study for coloring strength with the prediction models obtained with FT-Raman by Anastasaki et al. (*42*), even better results were achieved in the latter. Zalacain et al. (*43*) gave the NIR fingerprint spectra of saffron spice and compared it with that of a pool of crocetin esters obtained by column chromatography. Regarding origin differentiation, discriminant analysis among Iranian, Greek and Spanish saffron samples revealed that the Iranian saffron was the most different, whereas Greek and Spanish samples were more similar. Percent recognition for each group of samples was 100% for Iranian saffron, 95% approximately for Greek saffron, and 88% approximately for Spanish saffron. Fourier transform-Mid-infrared spectroscopy (FT-MIR) combined with multivariate analysis is another option that does not require complex sample preparation and obtains a fast spectral acquisition. FT-MIR has been applied for the discrimination of 250 saffron samples from Greece, Iran, Italy and Spain (*44*). The IR spectra of saffron filaments and their organic extracts were studied. The best discriminatory approach was achieved in the spectral region 2000–700 cm^{-1}. The spectral region responsible for the differentiation of Italian samples was in the region of the carbonyl group around 1746 cm^{-1}. This result was justified by the fact that Italian samples came from Sardinia where a process called "*feidatura*" takes place before drying the stigmas. In this process, the stigmas are wetted with extra virgin olive oil in order to improve the natural appearance of the stigmas as well as their preservation. The spectral region at around 1600 cm^{-1} that is assigned to the double bonds and the band at 1670 cm^{-1} that is attributed to the carbonyl functional group of safranal were responsible for the differentiation of samples from the remaining countries. The correct classification of 93.6% of the samples was achieved and the rates for saffron samples from Greece, Iran, Italy and Spain were 90.0, 89.5, 96.7 and 98.4%, respectively.

Table II. Amount of colorant necessary to make the adulteration detectable or to increase 10 units the coloring strength of a saffron solution (50 mg L^{-1}) and zone of the spectra where adulteration is detected (*34*, *35*)

Colorant	*Addition to increase 10 units the coloring strength (g colorant /kg saffron)*	*Detectable adulteration (g colorant /kg saffron)*	*Zone of the spectrum where the adulteration is detected (nm)*
Amaranth[a]	68.6	34.3	190-230; 510-590
Sunset yellow[a]	33.6	16.8	220-270
Yellow quinoline[a]	17.4	8.7	215-230/280-300
Azorubine[a]	80.6	8.1	190-230; 510-590
Betanine[a]	393.6	196.8	190-210; 300-400
Cochinela[a]	159.0	79.5	280; 500-600
Curcumin[a]	471.8	235.9	190-205
Erythrosine[a]	228.8	22.9	500-550
Naphtol yellow[a]	17.4	8.7	215-235
Norbixin[a]	14.6	n.d	n.d.
Ponceau 4R[a]	61.8	6.2	190-230; 510-570
Red 2G[a]	109.4	10.9	515-580
Allura red[a]	62.2	6.2	190-215; 515-580
Tartrazine[a]	18.0	n.d	n.d.
Crocin[a]		600	200-280; 310-350

Continued on next page.

Table II. (Continued). Amount of colorant necessary to make the adulteration detectable or to increase 10 units the coloring strength of a saffron solution (50 mg L^{-1}) and zone of the spectra where adulteration is detected (*34*, *35*)

Colorant	*Addition to increase 10 units the coloring strength (g colorant /kg saffron)*	*Detectable adulteration (g colorant /kg saffron)*	*Zone of the spectrum where the adulteration is detected (nm)*
Riboflavin[a]		50	190-250; 350-400
Chrysoidine G[a]		100	200-275; 330-400; 440-480
Rhodamine 6G[a]		5	500-590
Rhodamine B[a]		2	510-600
Safranin O[a]		20	200-300; 500-600
Alizarin Red S[a]		50	200-300
Carminic acid[a]		150	200-300; 500-590
Annatto[a]		50	420-490
Curcumin[b]		5	390-500
B-apo-8´-carotenal[b]		5	400-500
Alizarin[b]		10	200-300
Sudan I[b]		5	200-290/490-510
Sudan II[b]		5	200-290/490-520
Sudan Orange G[b]		5	390-490
Sudan Red[b]		2	490-600

Colorant	*Addition to increase 10 units the coloring strength (g colorant /kg saffron)*	*Detectable adulteration (g colorant /kg saffron)*	*Zone of the spectrum where the adulteration is detected (nm)*
Sudan III[c]		2	490-600
Sudan IV[c]		2	490-600
Annatto[c]		2	450-550
β-carotene[c]		5	400-510

UV-vis Spectra measured in water[a], acetonitrile[b], or chloroform[c]. n.d. = no detection.

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High Performance Liquid Chromatography

HPLC has demonstrated a great potential in saffron authentication. Nowadays, HPLC-DAD is the standard technique for the analysis of saffron components. However, HPLC-DAD on-line coupled with mass spectrometry (MS) is used when additional spectral information is required (*8*, *45–50*).

Figure 1B shows the characteristic HPLC chromatograms of saffron. At 440 nm the profile of crocetin esters can be checked, picrocrocin is determined at 250 nm and kaempferols and safranal at 330 nm.

ISO/TS 3632-2 test methods (*21*) include HPLC-DAD together with thin-layer chromatography (TLC) or as an alternative of TLC for identification of water-soluble acidic colorants. Before analyzing with HPLC, colorants are extracted, isolated and eluted using chromatography on a polyamide microcolumn.

HPLC can be used to fight against frauds with gardenia. A comparison between saffron and gardenia regarding the crocetin esters, picrocrocin and its related compounds determined by LC-ESI-MS was done by Carmona et al. (*30*). Their results showed that the aqueous extracts of saffron spice contain at least 15 different crocetin esters and can be differentiated from those of gardenia in the presence of the *trans*-crocetin di-(β-D-glucosyl) ester, by their greater proportion of the *trans*-crocetin (β-D-glucosyl)-(β-D-gentiobiosyl) ester and smaller proportion of the *cis*-crocetin di-(β-D-gentiobiosyl) ester and *trans*-crocetin (β-D-gentiobiosyl) ester. In the same analyses as those used for the crocetin esters, picrocrocin was characterized, along with related glycosylated compounds, with two new compounds from extracts of saffron being tentatively identified. Of all the compounds analyzed, the only one found in both saffron and gardenia was O-β-D-gentiobiosyl ester of 2-methyl 6-oxo-hepta-2,4-dienoic acid, which, together with the compound known as geniposide, makes up the chromatographic profile of gardenia at 250 nm. Thus, the case of adulterations with gardenia can be detected by the profile of crocetin esters together with the presence of compounds coming from gardenia that are not found in saffron, e.g. the geniposide, and the absence or low content in picrocrocin. Also the presence of mangicrocin, a xanthone-carotenoid glycosidic conjugated from saffron has previously been discussed as a biomarker for the control of authenticity (*51*).

The differentiation of saffron from different countries has been successfully carried out in several studies using HPLC measures of the chemical compounds present in the saffron matrix. Carmona et al. (*15*) characterized the flavonoid fraction in saffron spice by LC-DAD/ESI-MS/MS and underlined the ability of kaempferol glycosides to act as markers for the origin of saffron spice. They found significant differences in these flavonoids contained in saffrons from Spain, Greece, Iran and Morocco. A discriminant analysis separated the samples by their respective kaempferol-3-*O*-sophoroside contents. Also in order to determine origin, the free amino acids and ammonium ion profile of saffrons from Greece, Iran, Italy and Spain were studied (*16*). The results were reported as useful for saffron origin differentiation and the analysis of free amino acids and ammonium has therefore been proposed as a rapid tool to differentiate European from Iranian samples.

Paper and Thin-Layer Chromatography

Sampathu et al. (*25*) summarized the position and color of spots corresponding to saffron and some adulterants such as curcuma or safflower in paper chromatography and TLC methods for detection of adulterations. Other TLC procedures in saffron have been reported (*50*, *52*).

TLC is also proposed in ISO/TS 3632 (*21*) for the identification of main components after soaking the saffron in methanol. It reveals the pigments existing in saffron, picrocrocin, β-hydroxycyclocitral and safranal. Consequently, it may be used as an indicator of the authenticity of the product. In this Technical Specification, another test using TLC for the detection and identification of artificial water-soluble acidic colorants after their isolation and elution on a polyamide microcolumn is proposed. More recently, Pathan et al. (*53*) have developed and validated a high-performance thin-layer chromatographic method for the quantitative analysis of safranal as bulk, in saffron extract and in a developed safranal-loaded nanoparticle formulation.

Gas Chromatography

GC also offers a fingerprint of saffron to detect adulterations and a tool for geographical differentiation.

Alonso et al. (*26*) established the fingerprint of Spanish saffron aroma through the analysis of 252 samples. The authors also demonstrated that thermal desorption (TD) GC-MS was an adequate technique for detecting adulterations with safflower and with synthetic safranal. Kanakis et al. (*54*) characterized the volatile constituents of Greek saffron and determined its content of safranal and HTCC, with respect to the drying process and storage period at 4 °C by GC-MS and GC-FID analysis. In this study, the isolation of the volatile constituents was performed using ultrasonic assisted extraction (USAE) and microsimultaneous hydro distillation extraction. USAE-GC-MS was also applied by Jalali-Heravi et al. (*55*) to establish a fingerprint for Iranian saffron. In this study the resolution and quantification of unresolved GC-MS signals were achieved by using self modelling curve resolution (SMCR) techniques.

Carmona et al. (*6*) used GC-MS to determine volatile markers of saffron and an electronic nose based on metal oxides to determine the volatile profile of the samples from different geographic origins. The authors demonstrated by means of TD-GC-MS that 3,5,5-trimethyl-2-cyclohexenone, 2,6,6-trimethylcyclohexane-1,4-dione and acetic acid are capable of differentiating saffron from its origin. They found very high contents of acetic acid in Iranian and Moroccan samples, while low or undetectable content was observed in Greek and Spanish samples. By using an e-nose specifically optimised for this application and Principal Component Analysis (PCA), they were able to discriminate samples from different countries with a 90% degree of certainty. The authors attributed the results to the different postharvesting processes that saffron undergoes depending on its origin.

Anastasaki et al. (*56*) showed the potential of saffron volatiles to discriminate geographical origin (247 saffron samples from Iran, Greece, Spain and Italy) using USAE for isolation, GC-MS/FID for analysis and discriminant analysis.

Maggi et al. (*57*) used USAE with an organic solvent and dynamic headspace desorption (DHD) followed by GC-MS to screen for commercial saffron volatile composition. They analyzed the aroma quality of 418 saffron samples available on the market to consumers and belonging to different ISO categories, finding that USAE allowed for the detection of a greater number of compounds and differentiation of ISO categories, whereas DHD was faster, required a smaller amount of saffron and was able to characterize the saffron volatile fingerprint.

Maggi et al. (*58*) studied how saffron volatile profiles and its sensory characteristics change during storage. They analyzed 73 samples with different storage times (<1 year, 3–4 and 8–9 years) in order to determine aromatic descriptors obtained by gas chromatography–olfactometry (GC–MS/FID-O) and related them with overall saffron chemical volatile composition analyzed by USAE–GC–MS. Safranal and other major compounds such as 4-hydroxy-2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde and 3,5,5-trimethyl-2-cyclohexene-1-one, were significantly different for less than 1 year of storage when compared with the 3–4 and 8–9 years of storage, although the minor constituents 2-hydroxy-3,5,5-trimethylcyclohex-2-en-1,4-dione and isomer of 4-hydroxy-3,5,5-trimethyl-2-cyclohex-1-one varied significantly for all three harvests. Saffron with less than 1 year of storage contained a higher proportion of saffron, flower and spicy descriptors, while the oldest saffrons (3–4 and 8–9 years of storage) contained volatiles with vegetal, caramel and citrus notes. The aromatic notes that contributed most to saffron storage differentiation were spicy, freshly cut grass and vegetable. Although little research has been done, this study could be considered as a first step in the authentication of saffron age.

Nuclear Magnetic Resonance (NMR)

The ^{1}H-NMR spectrum of *trans*-crocetin di-(β-D-gentiobiosyl) ester was reported by Speranza et al. in 1984 (*59*).

Yilmaz et al. (*60*) have recently reported the feasibility of using unsupervised PCA models of ^{1}H NMR metabolic fingerprints to assess the authenticity of saffron. The authors explored the ^{1}H NMR metabolic fingerprints of 31 authentic reference saffron samples from Iran, and 32 samples that had been purchased from retail stores in several countries.

The results showed that the Iranian reference samples were very similar and clustered tightly together to apparently authentic commercial saffron samples. Disparate commercial spice samples that did not seem to be real saffron were readily identified. Some of these samples had a distinct profile resembling a synthetic mixture rather than an extract of saffron or contained other plant material, as confirmed by subsequent microscopic analysis. Differences in production sites and drying conditions of the authentic Iranian samples were not reflected in the metabolic fingerprints. In this study, by using statistical correlation spectroscopy, picrocrocin and crocetin esters emerged as the most important ^{1}H NMR markers of authentic saffron.

Isotope Analysis

Carbon stable isotope analysis was applied trying to classify saffron according to the isotope ^{13}C content of the safranal contained in each sample (*61*). The objective was not achieved, although the authors managed to distinguish between one sample of safranal extracted from saffron and one sample of safranal produced by chemical synthesis through the site specific $^{2}H/^{1}H$ ratio analysis of safranal by SNIF-NMR™. Significant differences in the $^{2}H/^{1}H$ ratio were observed at each of the six molecular environments measured by SNIF-NMR. Semiond et al. (*62*) also studied the distinction between safranal extracted from saffron and synthetic safranal using carbon stable isotope analysis. They reported the successful discrimination of one synthetic safranal sample and 5 samples of safranal extracted from saffron using methanol and super critical fluid extraction. However, the $\delta^{13}C‰$ values of extracted safranal differed significantly from those previously reported (*61*). Semiond et al. (*62*) went on to discuss the use of the measured $\delta^{13}C‰$ values of safranal to distinguish geographical origin of saffron. They concluded that carbon isotope ratio analysis did not provide the means to distinguish the geographical origin of saffron.

Nevertheless, a recent study (*63*) has proved that analysis of the stable isotopes of the bio-elements H, C and N of saffron from production areas of Western Macedonia in Greece, Khorasan in Iran, Sardinia in Italy and Castilla-La Mancha in Spain combined with multivariate analysis is a reliable tool to discriminate the geographical origin of saffron. Further work is proposed by the authors to establish the long-term stability of the reported models with respect to different harvests and including other major producers such as India and Morocco.

Molecular Genetic Analysis

A distinction between saffron and its adulterants or substitutes: *Carthamus tinctorius* L., *Hemerocallis fulva* L. and *Hemerocallis citrina* Baroni, via molecular genetic analysis was drawn by Ma et al. (*64*). The spacer domains of 5S-rRNA were cloned from the genomic DNAs of these plants. The cDNAs encoding the spacer domains, about 300 to 500 bp, were sequenced. The authors found great diversity in the nucleotide sequences of these four species, which could serve as markers for authenticity of saffron and differentiation from substitutes and counterfeits.

Perspectives Concerning Saffron Authentication

As in other foods, instead of just one method, a combination of results from multiple methods, e.g chemical fingerprints, isotope ratio profile, and genetic information, along with statistical tools will be necessary to authenticate saffron completely. Recent advances together with the knowledge about this spice generated over centuries make it possible to guarantee that consumers are actually getting what they are paying for. Nevertheless, adulterers are always one step ahead, and authorities and analysts must be alert to ensure the quality and safety of saffron. In this sense, the possible appearance of new coloring matters will prompt

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analysts to do research on new methods of detection. Moreover, the panorama of saffron production has been shifting in recent years. In many countries there is a growing interest in saffron production due to the low economic yields of other agricultural products. Traditional cultivation techniques will be changed by the application of mechanized production techniques and forced flowering. These new ways of production, although on a small scale, are realities in countries such as China, Chile or Spain, which leads us to believe that new forms of saffron, applications and parameters to define categories will be available in a not so distant future.

This new scenario will undoubtedly demand innovative analytical methods as regards authentication.

In the end, all production steps, from field to consumer, will have to be taken into account in order to guarantee authenticity of saffron.

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Chapter 23

Multiresidue Pesticide Analysis of Ginseng and Other Botanical Dietary Supplements

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Multiresidue pesticide procedures utilizing salt-out organic solvent extraction, clean-up by solid-phase extraction or dispersion, and analysis by capillary gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography-mass spectrometry (LC-MS) have been developed for ginseng and other botanical dietary supplements. The methods were validated for over 350 pesticides. LOQs of 10-20 and < 15 μg/kg have been obtained in ginseng by GC- and LC-tandem mass spectrometry (GC-MS/MS and LC-MS/MS), respectively. Recoveries were 70-120% and RSDs < 20% for most of the pesticides studied. These procedures allow for effective pesticide monitoring in botanicals at low concentrations. The goal of this work is to present strategies that would lead to cost-effective and efficient multiresidue pesticide analysis of botanical dietary supplements based on pesticide type regardless of the matrix.

Keywords: multiresidue pesticide analysis; botanical dietary supplements; gas chromatography-mass spectrometry; high performance liquid chromatography-mass spectrometry

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Introduction

The increasing risks to human health generated by the widespread use of pesticides in our environment and food supply are well established. To ensure those risks are low, food is routinely monitored by the U.S. Food and Drug Administration (FDA) for purity and compliance to established regulations and tolerances (Federal Food, Drug, and Cosmetic Act (FFD&C Act), 21 U.S.C. 342(a)(2)(b)). In 1994, Congress amended the FFD&C Act with the passage of the Dietary Supplement Health and Education Act of 1994 (DSHEA) (Pub. L. 103-417) (*1*). This law established a new paradigm for the regulation of dietary supplements (*2–4*). Among other things, DSHEA defined dietary supplements to include certain products that contain herbs and botanicals (21 U.S.C. 321(ff)). DSHEA also provided the FDA with the authority to establish good manufacturing practice requirements to govern the preparation, packaging, and holding of dietary supplements and to ensure that these products are not adulterated.

Botanical supplements are used by consumers to improve their health, energy and vitality. Botanical ingredients that may be used in dietary supplements have been used for centuries in Asia and Europe and their use in the United States is on the rise (*5*). Since many of these botanicals are farmed by conventional agricultural practices which include pesticide application, there is evidence that some botanical ingredients used in dietary supplements may be contaminated with pesticide residues (*6–20*). To improve on the detection of pesticide contaminants in dietary supplements and to ensure their safety and quality, validated pesticide analytical methods are needed for analyzing pesticide residues in dietary supplement products. There is current interest regarding the presence of pesticides in botanical dietary supplements as indicated by studies by other groups (*6*, *8*, *10*, *11*, *13–16*, *18–20*). Government agencies, industry, health communities, and consumer groups require information to evaluate the scope or nature of these potential problems and to determine the necessary compliance or regulatory standards. If there are no maximum amount or tolerance of residues established, botanical dietary supplements containing pesticides are considered adulterated according to the FFD&C Act. A raw agricultural commodity or processed food or feed is deemed adulterated if a pesticide chemical residue for which no tolerance has been set is present.

Due to the necessity of multiresidue pesticide methods for botanical products, the U.S. Food and Drug Administration (FDA) has been working on validated procedures to analyze, quantitate and qualitate a wide range of pesticides in botanical matrices. One procedure involves the modification of existing methods commonly used for fresh plant foods and utilizing gas chromatography coupled with tandem-mass spectrometry (GC-MS/MS) for the analysis of semivolatile pesticides, primarily organohalogen, organophosphorus and pyrethroid pesticides (*11*). This procedure has been successfully applied to the analysis of 167 pesticides to ginseng powders and MS/MS was shown to be more specific, sensitive and effective than GC-MS in selective ion monitoring mode (GC-MS/SIM). A second method, utilizing high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS), is necessary for the analysis of thermally labile and polar pesticides, which can degrade and therefore are not amenable to the high

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temperature conditions used in GC analysis (*12*). Therefore, these rugged and effective GC and LC methods are essential and required for the determination of pesticides in botanical products, for surveillance and regulatory purposes to ensure the safety and wholesomeness of the product.

Methods and Materials

The complete details of the materials and procedures are described elsewhere (*11*, *12*) but the methods are summarized in Figure 1(A) and (B) for GC-MS and LC-MS analysis, respectively. In general, the LC-MS/MS analysis involves the original *Qu*ick, *E*asy, *Ch*eap, *E*ffective, *R*ugged and *S*afe (QuEChERS) procedure developed by Anastassiades *et al.* (*21*), which involves salt-out acetonitrile extraction followed by a clean-up step using solid-phase dispersion containing anhydrous $MgSO_4$ and primary-secondary amine (PSA) sorbent. The dried botanical (1.0 g) is mixed with 10 mLs of water, 10 mLs of acetonitrile (1:1 water:acetonitrile) and 200 µL of a mixture of isotopically-labeled internal standards, D_{10}-diazinon, D_6-malathion, D_6-dichlorvos, D_6-linuron, and D_6-diuron (450-700 µg/mL prepared in acetonitrile). Pesticides are extracted by adding 4.0 g anhydrous magnesium sulfate and 1.0 g sodium chloride to the slurry, followed by vigorous shaking and centrifugation to induce a phase separation between the aqueous and organic acetonitrile layers. The acetonitrile extract is removed and cleaned up by transferring the extract to a test tube consisting of 300 mg anhydrous magnesium sulfate and 150 mg PSA sorbent. The tube is shaken, centrifuged, and 400 µL of the extract is mixed 100 µL acetonitrile and 500 µL 10 mM ammonium formate and filtered into autosampler vials using a 0.2 µicron Nylon membrane syringe. Matrix-matched calibration standards used for quantitation were prepared by replacing the 100 µL acetonitrile with calibration standards prepared in acetonitrile and using 400 µL of botanical extracts that had been tested to be pesticide-free. Pesticides were analyzed using high performance liquid chromatography interfaced with a hybrid quadrupole-linear ion trap mass spectrometer through a positive electrospray interface and operating in tandem (MS/MS) mode. Two selected reaction monitoring (SRM) transitions were monitored for both the native and stable isotopically labeled compounds and are provided elsewhere (*12*).

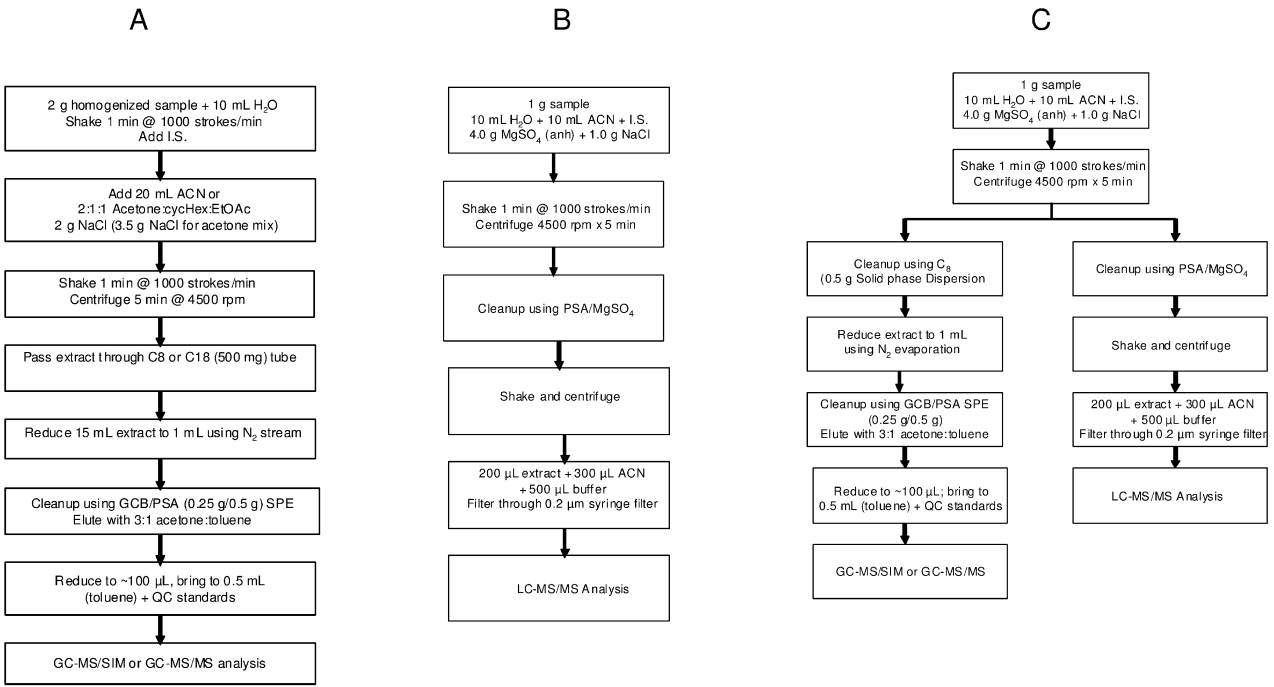

*Figure 1. Flowcharts for multiresidue procedures to analyze pesticides in dried botanical dietary supplements using (**A**) GC-MS, (**B**) LC-MS/MS and (**C**) combination of GC-MS and LC-MS techniques.*

For GC-MS analysis, 1.0 g of sample was mixed with the acetonitrile:water (1:1) and 200 µL of internal standard solution, 3.4 µg/mL tris(1,3-dichloroisopropyl) phosphate and mixed with 4.0 g anhydrous magnesium sulfate and 1.0 g sodium chloride. The sample is shaken vigorously and centrifuged and the extract is transferred to a 50 mL disposable centrifuge tube containing 500 mg C18 sorbent. The tube is shaken and centrifuge and the resulting extract is reduced to ~1 mL using a gentle N_2 stream in a 55 °C water bath. The concentrated extract was cleaned up using solid-phase extraction (SPE). The SPE cleanup procedure involved a tandem cartridge consisting of a dual layer sorbent of 250 mg graphitized carbon black (GCB, top) and 500 mg PSA sorbent (bottom) and topped with approximately 250-500 mg anhydrous sodium sulfate. The SPE cartridge was conditioned with three column volumes of acetone, followed by loading sample extract and elution with 12 mL of 1:3 toluene:acetone solution. The eluate was reduced to 100 µL using a gentle N_2 stream and a 55 °C water bath to evaporate the excess solvent for sensitivity needs or solvent exchange. To the extract, 500 µL of toluene and a quality control standard consisting of deuterated polycyclic aromatic hydrocarbons (25 µL of 20 µg/mL solution) were added. The extracts were analyzed for pesticides using capillary gas chromatography chromatography interfaced with a single quadrupole or triple quadrupole mass spectrometer through electron impact ionization and operating in selective ion monitoring (GC-MS/SIM) or tandem (GC-MS/MS) mode, respectively. Three or four selected ions in SIM mode or two selected reaction monitoring (SRM) transitions were monitored for the pesticides, internal standard, and stable isotopically labeled compounds in GC-MS/SIM or GC-MS/MS modes and the data are provided elsewhere (*11*).

Results and Discussion

GC-MS Analysis of Pesticides in Botanicals

The method used for the GC-MS analysis of pesticides is outlined in Figure 1A. Salt-out organic solvent extraction is a common procedure used to extract and partition nonpolar pesticides from a plant material into an organic solvent, such as acetonitrile (*22*, *23*), acetone (*24*), or ethyl acetate (*25*). The addition of water has also been shown to aid in the extraction of pesticides from dehydrated plant materials. The clean-up procedures in this work employ C-8 or C-18 solid-phase dispersive cleanup, followed by solid-phase extraction using tandem GCB/PSA cartridges. These sorbents have been shown to remove organic acids and plant pigments such as chlorophyll, carotenoids, polyphenols and sterols, which can potentially contribute to matrix-enhancement effects in capillary GC detection. However, GCB can also retain structurally planar and aromatic pesticides and metabolites such as hexachlorobenzene, chlorothalonil, and pentachlorobenzene and requires toluene to elute the bound pesticides from the sorbent (*16*, *22*, *23*).

Figure 2 illustrates the advantage of GC-MS/MS (Figure 2A) over GC-MS/SIM (Figure 2B) in terms of specificity and sensitivity in the separation of the α-, β-, δ-, γ- and ε- BHC isomers. Although extensive SPE clean-up procedures are used, the ginseng matrix is relatively concentrated and complex

such that many of the pesticides, particularly polar organophosphorus pesticides and early eluting pesticides, are difficult to detect and identify by GC-MS/SIM. The matrix components in the botanical extracts can contribute to the analyte's target and qualifier ion abundances and affect the qualifier-to-target percentage ratios used for identification in GC-MS/SIM, as in the case of the β- and γ-BHC isomers shown in Figure 2B. The specificity of GC-MS/MS involves the use of transition ions that are specific to the pesticide even in the presence of the ginseng matrix, which is illustrated in the complete separation of all 5 BHC isomers in Figure 2A. The specificity of MS/MS results in an improvement of the signal-to-noise ratio which allows for improved sensitivity over MS/SIM, and results in an increase of pesticides detected and identified at lower concentrations. As shown in Figure 3, most of the pesticides found in incurred ginseng samples can easily be identified and quantitated by both GC-MS/MS (Figure 3A) and GC-MS/SIM (Figure 3B) methods. The pesticides analyzed tend to be persistent organochlorine pesticides, such as DDT, quintozene, and their metabolites. The results show that GC-MS/SIM can be used in screening for a limited number of pesticides in ginseng, provided that the matrix components in ginseng do not interfere with the analyte's SIM ions.

Standard mixes of organohalogen, organophosphorus and pyrethroid pesticides, including isomers and metabolites in solvent were used to determine retention times, mass spectra and acquisition time segments for both GC-MS/SIM and GC-MS/MS. Matrix-matched standards ranging in concentrations from 5.0 to 5000 ng/mL were used to determine linearities and the limits of quantitation (LOQs) of each pesticide. Most of the pesticides can be determined in this range ($r^2 > 0.99$, n=10) when analyzed by GC-MS/MS. The linearities for pesticides analyzed by GC-MS/SIM differ for each pesticide because of their different LOQs, which ranged from 5 to 333 ng/g. The geometric mean LOQs based on the LOQs of 168 pesticides, metabolites and isomers and analyzed by GC-MS/SIM and GC-MS/MS were determined to be 53 and 6.6 ng/g, respectively. Most of the LOQ values for GC-MS/MS were typically in the 1-20 ng/g range, compared to 83 - 167 ng/g for GC-MS/SIM, indicating that GC-MS/MS is more sensitive than GC-MS/SIM.

The procedure was validated using fortification studies in different botanical matrices. Figures 4 and 5 list the mean recoveries and relative standard deviations (RSDs) (n = 4) for approximately 300 pesticides fortified at the 10, 25, 100 and 500 ng/g of dry weight of ginseng, black cohosh root, Astralagus root, and St. John's Wort and analyzed by GC-MS/MS. Recoveries for most of the pesticides in the four matrices were in the 70-100% range and RSDs were typically less than 20%, which are acceptable values for method validation. Increased variations in the average recoveries and RSDs for the pesticides were observed at the lower fortification levels (10 and 25 ng/g).

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ACS Symposium Series; American Chemical Society: Washington, DC, 2011.

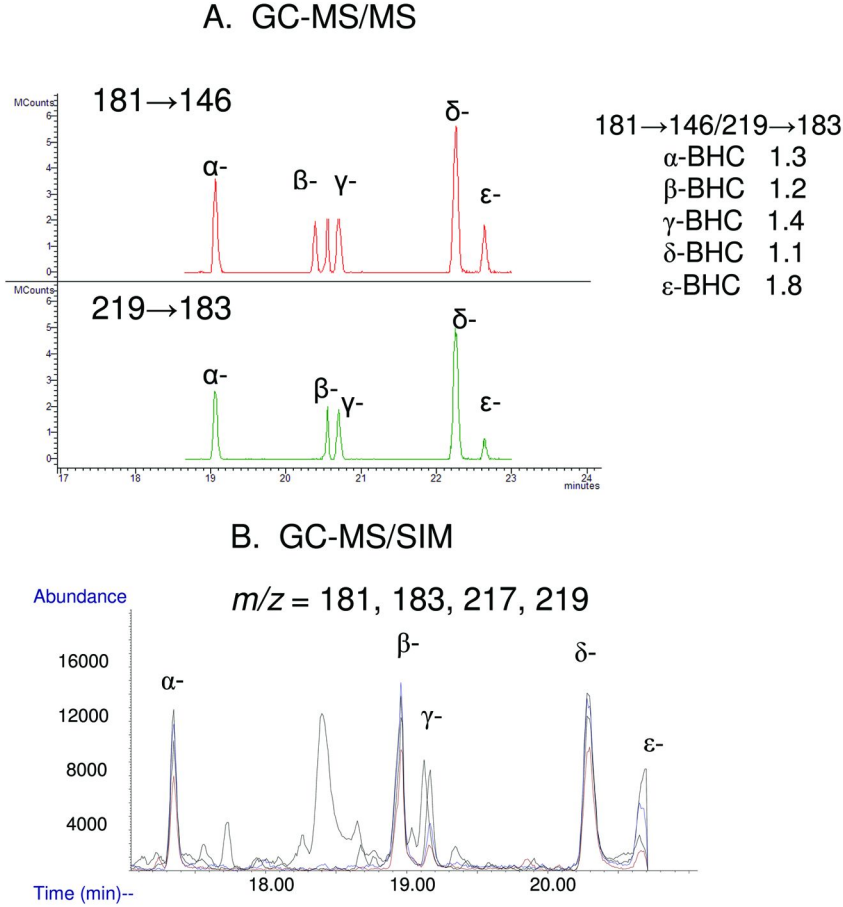

*Figure 2. Presence of incurred BHC residues in dried ginseng powder (Panax quinquefolius) determined by acetonitrile salt-out extraction, cleaned up by GCB/PSA solid-phase extraction and analyzed by (**A**) GC-MS/MS and (**B**) or GC-MS/SIM. For GC-MS/MS (**A**) analysis reveals the presence and separation of α-, β-, γ-, δ-, ε-BHC isomers by the two transitions, 181 → 146 (primary, quantitation) and 219 → 183 (secondary, qualifier). Values of 181 → 146/219 → 183 are provided to show that the ratios of the four isomers resulting from the two extraction solvents are similar. The ε-BHC ion ratio was not determined In (**B**), GC-MS/SIM indicates interferences in the screening and identification of the β- and γ-BHC isomers using m/z 181, 183, 217, 219.*

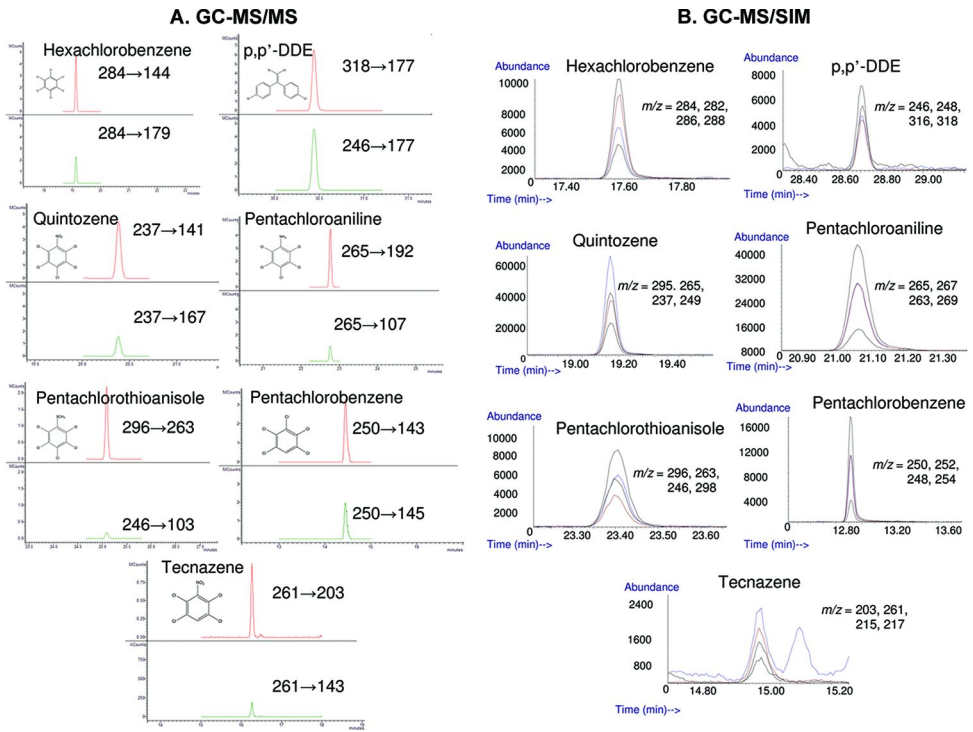

*Figure 3. Presence of incurred pesticides and metabolites in the same dried ginseng sample as in Figure 2 determined by (**A**, left panels) GC-MS/MS and (**B**, right panels) GC-MS/SIM. Incurred pesticides present in the ginseng samples are hexachlorobenzene, p,p'-DDE, quintozene, pentachloroaniline, pentachlorothioanisole, pentachlorobenzene, and tecnazene.*

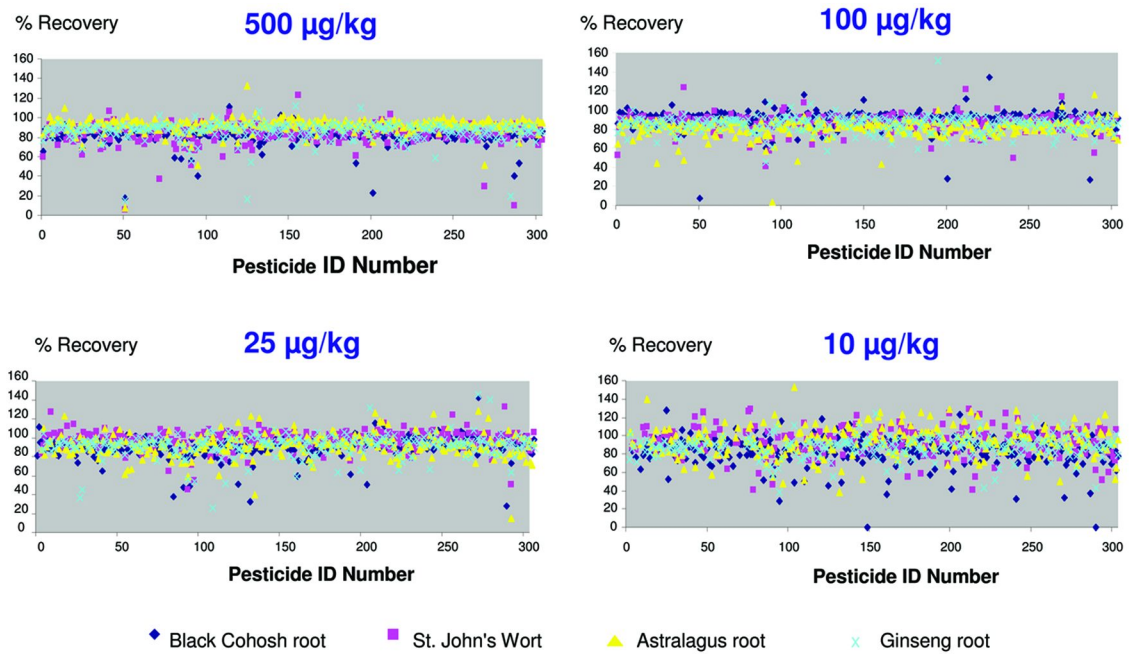

Figure 4. Average recoveries of approximately 300 pesticides in astralagus root, black cohosh root, ginseng root, and St. John's wort using acetonitrile salt-out extraction and solid-phase extraction cleanup determined at four fortification levels, 10, 25, 100 and 500 µg/kg (n = 4), followed by GC-MS/MS analysis. Most of the recoveries are within the 70-100% range, indicating the accuracy of the method.

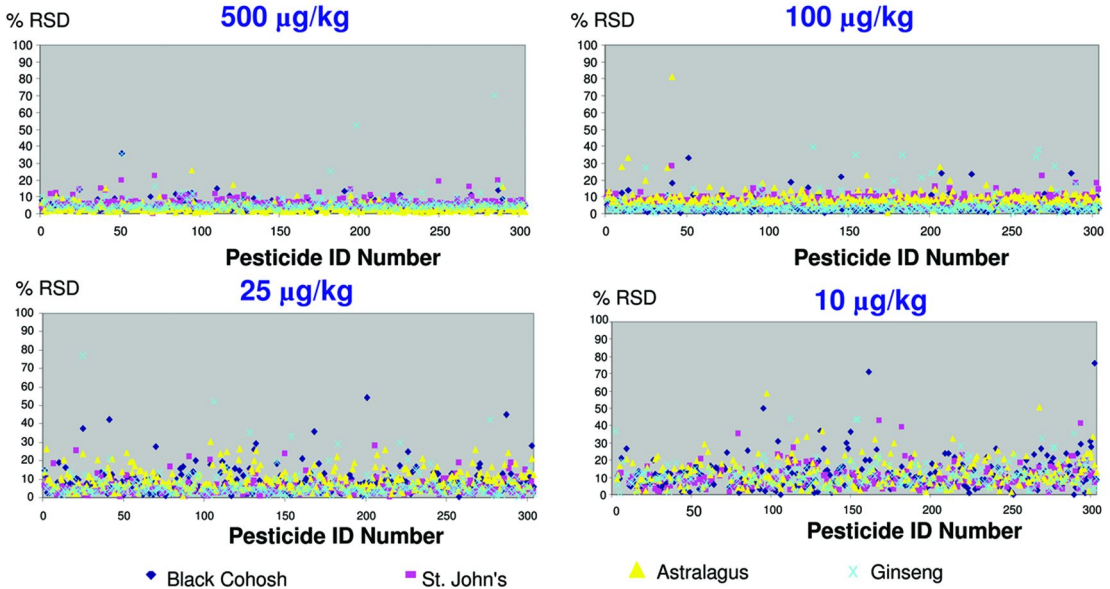

Figure 5. Relative standard deviations (%) of approximately 300 pesticides in astralagus root, black cohosh root, ginseng root, and St. John's wort using acetonitrile salt-out extraction and solid-phase extraction cleanup determined at four fortification levels, 10, 25, 100 and 500 µg/kg (n = 4), followed by GC-MS/MS analysis. Most of the %RSDs are less than 20% , indicating the precision of the method.

The sample preparation procedure and analysis by GC-MS were shown to be effective and used to analyze incurred ginseng samples. The common pesticides found in five different ginseng samples were quintozene and its metabolites, pentachloroaniline, pentachlorobenzene and pentachlorothioanisole and other contaminants found in technical-grade quintozene, such as hexachlorobenzene, tecnazene, and 2,3,5,6-tetrachloroaniline. The structures of these pesticides and metabolites and the concentration levels found are shown in Figure 6. Other pesticides listed in Figure 6 include organochlorine fungicides and insecticides, such as DDT and its metabolites, the isomers of BHC, procymidone, 2-phenylphenol, chlorothalonil, dacthal, and the two organophosphorus insecticides, chlorpyrifos and diazinon. The concentrations of these compounds range from detected levels of chlorothalonil and dacthal to 1770 μg/kg of quintozene. Two other fungicides, azoxystrobin and dimethomorph have also been found at < 20 μg/kg in some of the ginseng samples. The presence of banned organochlorine insecticides, such as *p,p′*-DDT and its metabolites and BHC isomers, is probably due to the persistence of these pesticides in the environment that the ginseng roots were cultivated in. The presence of procymidone, quintozene and quintozene by-products indicates the effectiveness, availability and the technical quality of these fungicides in controlling mold growth of these valued root commodities.

LC-MS/MS Analysis of Pesticides in Botanicals

The method used for LC-MS/MS analysis is outlined in Figure 1B. The sample preparation procedure used is simpler than the GC-MS procedure in Figure 1A because LC-MS is tolerant to larger sample sizes and has better sensitivity. The Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) procedure (*21*) used for fresh produce was modified for dried botanical products by the addition of water. This is essentially a salt-out acetonitrile partition followed by a solid-phase dispersive clean-up with primary-secondary amine (PSA) sorbent. The detection limits using the QuEChERS procedure and LC-MS/MS analysis have been determined for approximately 200 pesticides elsewhere (*12*) in ginseng and are in the range of 0.07 - 15 μg/kg.

Botanicals vary based on chemical composition and in addition to ginseng root, two other botanicals, gingko biloba leaves and saw palmetto berries were also evaluated. The composition differences in botanicals make it difficult to develop a multiresidue pesticide method for botanicals. QuEChERS with LC-MS/MS has undergone collaborative validation as a multiresidue pesticide method in fresh produce (*26*) and a single laboratory validation is being extended to dried botanical products in this work. Figure 7 reveals the average recoveries and relative standard deviations of ginseng root, Gingko biloba leaves, and saw palmetto berries at three fortification levels of 25, 100 and 500 μg/kg. The average recoveries for most of the pesticides are between 80-120% and RSDs < 20% for most of the pesticides in the three botanical matrices. The results of this study indicate that the procedure is adequate for dried botanical products and the procedure can be used to analyze ginseng samples for pesticides that may not be amenable to GC or GC-MS analysis.

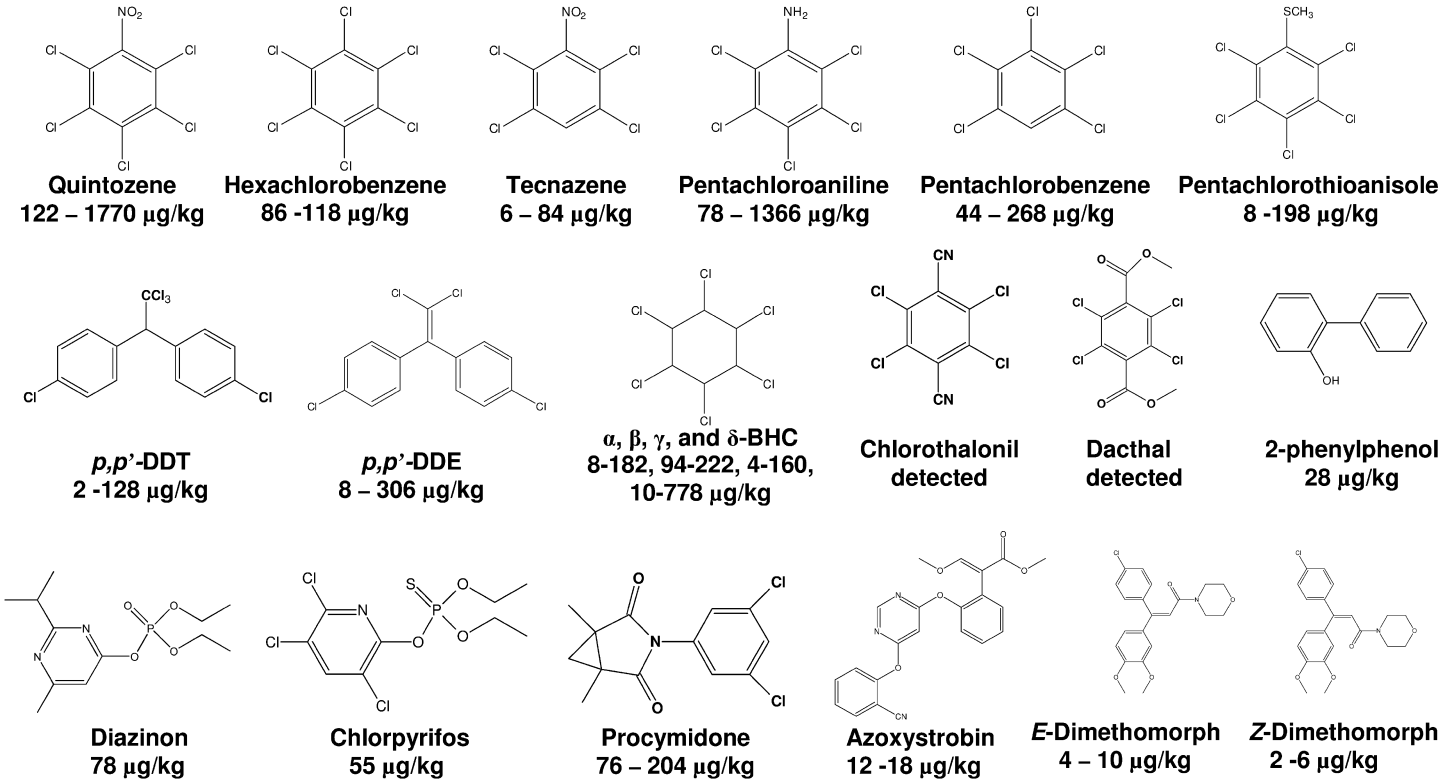

Figure 6. Pesticides and pesticide metabolites found in ginseng samples including concentrations or concentration ranges.

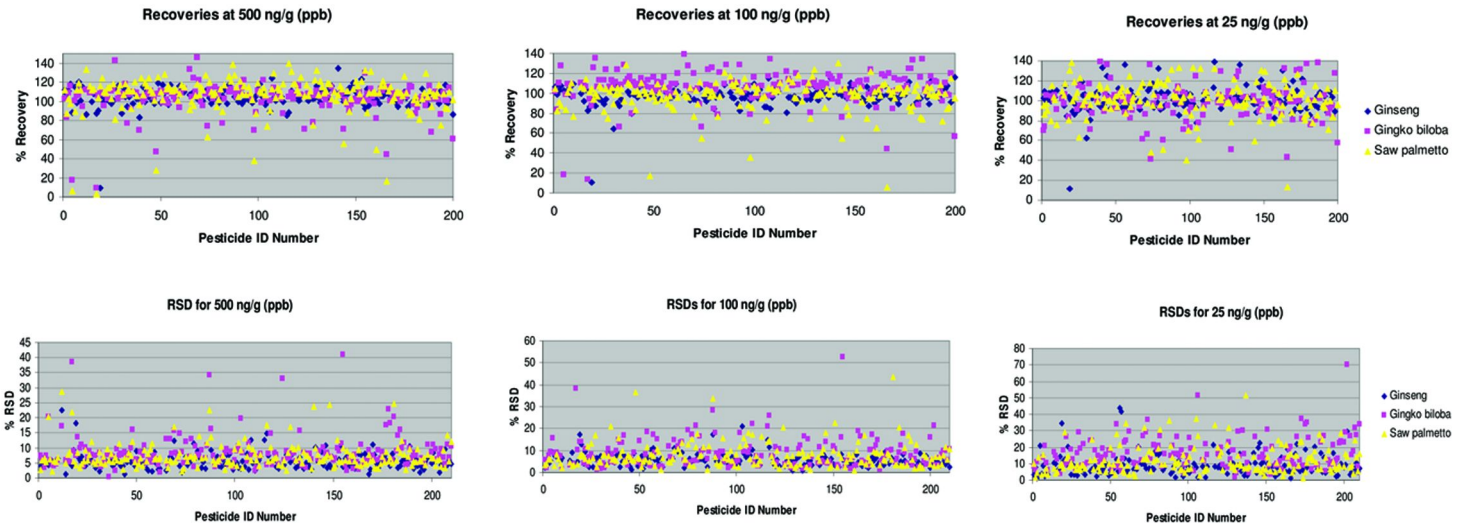

Figure 7. Average recoveries and relative standard deviations (%) of approximately 200 pesticides in Gingko biloba leaves, ginseng root, and saw palmetto berries using the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) procedure determined at three fortification levels, 25, 100 and 500 µg/kg (n = 4), followed by LC-MS/MS analysis. Most of the recoveries are 80-120% and RSDs < 20%, indicating the accuracy and precision of the method can be used for pesticide analysis of botanical samples.

Figure 8 shows LC-MS/MS chromatograms of a single ginseng sample found to contain seven pesticides. Many of these pesticides shown can be analyzed by GC and GC-MS techniques but are better detected by LC-MS/MS due to improved sensitivity (*27*). Most of the pesticides found are no longer the traditional organochlorine, organophosphorus and pyrethroid pesticides but are part of a newer generation of pesticides such as conazole (etaconazole, flusilazole), anilide (metalaxyl and oxadixyl), and strobilurin (azoxystrobin) fungicides that are currently being used for pesticide applications in conventional agricultural practice. LC-MS/MS optimizes analysis of samples because the screening, quantitation, and identification can be achieved in a single injection when two ion product transitions are used (*28*). In the case of the ginseng sample in Figure 8, the top transition is typically used for quantitation because of its higher abundance compared to the bottom transition, which is primarily used as a confirmatory ion for pesticide identification.

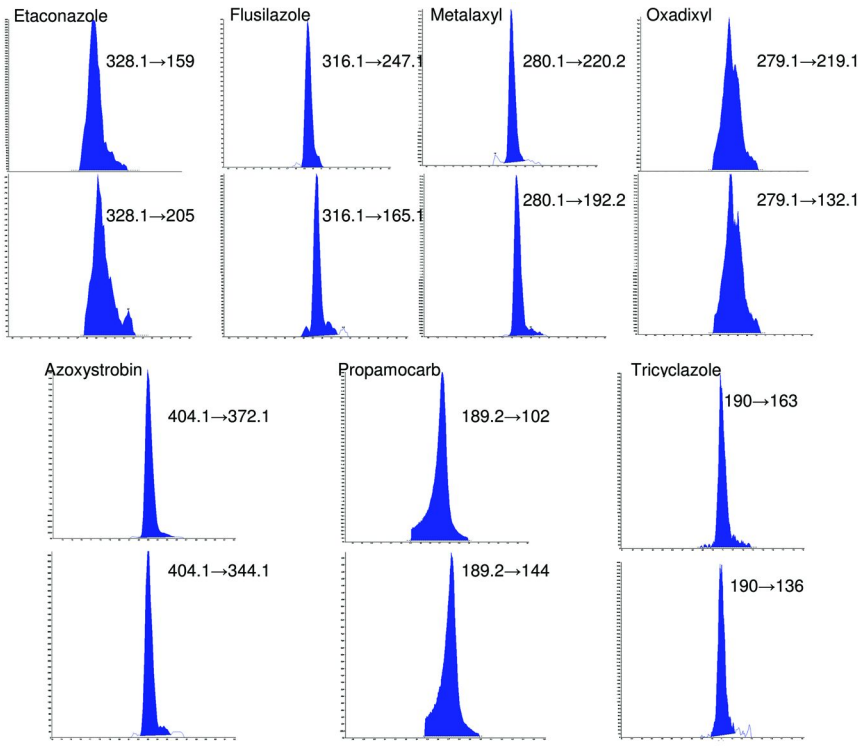

Figure 8. LC-MS/MS chromatograms of seven pesticides found in a ginseng sample. Incurred pesticides present in the ginseng sample are etaconazole (328.1→159, 328.1→205), flusilazole (316.1→247.1, 316.1→165.1), metalaxyl (280.1→ 219.1, 280.1→192.1), oxadixyl (279.1→219.1, 279.1→132.1), azoxystrobin (404.1→372.1, 404.1→344.1), propamocarb (189.2→102, 189.2→144), and tricyclazole (190→163, 190→136). Pesticides were determined using the two ion transitions for quantitation and identification.

Table 1 lists the concentrations of all seven pesticides ranging from azoxystrobin (0.5 ± 0.1 μg/kg) to propamocarb (112 ± 9 μg/kg), determined by the target (or quantitation) transitions (i.e., 404.1→ 372.1 and 189→102 for azoxystrobin and propamocarb, respectively). The identification of the pesticides is determined by calculating the peak area ratio of the two transitions (i.e., 404.1→372.1/404.1→344.1 and 189.2→102/189.2→144 for azoxystrobin and propamocarb, respectively) (*27*). Table 1 compares the ion transition ratios between the pesticides found in the ginseng sample with the ion ratios of calibration standards of the concentrations close to the concentrations of the pesticide found in the sample. For the identification of the seven pesticides, there is sufficient agreement within 20% between the ion ratios of the sample to that of the calibration standard.

Table 1. Pesticides found in a ginseng sample analyzed by LC-MS/MS (Figure 8), their determined concentrations (ppb, *n* = 3), and ion ratios of the sample compared to calibration standards at the appropriate concentration level. The procedure indicates the presence and identification of the pesticide in the ginseng sample.

	Incurred		*Standard*	
Pesticide	*Conc (ppb)*	*Ion ratio*	*Conc (ppb)*	*Ion ratio*
Azoxystrobin	0.5 ± 0.1	10	0.67	11
Etaconazole	5.0 ± 0.5	1.81	6.67	1.89
Flusilazole	1.0 ± 0.02	1.31	1.67	1.26
Metalaxyl	3.5 ± 0.2	1.43	3.33	1.43
Oxadixyl	40 ± 2	3.24	33.3	2.99
Propamocarb	112 ± 9	3.61	80	3.58
Tricyclazole	0.8 ± 0.04	1.04	0.67	0.97

GC-MS and LC-MS/MS Analysis

To screen for a wide variety of pesticides, both GC-MS and LC-MS procedures shown in Figure 1A and 1B can be combined to form a comprehensive procedure (Figure 1C). Many of the organochlorine, pyrethroid, and non-polar pesticides are poorly ionized by atmospheric pressure ionization (API) techniques typically used in LC-MS and require electron impact (EI) ionization and GC-MS techniques for ionization of the pesticide and better detection. Since many of these organochlorine pesticides such as DDT and BHCs are persistent, they can be better detected by GC-MS/MS in root botanicals such as ginseng. Other organochlorine fungicides are efficacious in the prevention of mold growth of these valuable commodities so the need for GC-MS techniques still remains since quintozene and its metabolites, pentachloroaniline, pentachlorobenzene, pentachlorophenyl methyl ester and the contaminant such as hexachlorobenzene,

do not ionize well under electrospray ionization in LC-MS. However, the use of GC-MS does require additional clean-up steps since sample introduction to the instrument is limited. Since the production of botanicals follows similarly to conventional agricultural practices such as pesticide applications and use, the detection of new classes and generation of pesticides are better suited for LC-MS analysis.

Future work will involve adopting and improving current multiresidue procedures for fresh agricultural commodities to botanical dietary supplements. The wide diversity of botanicals requires extensive validation studies to ensure that methods are rugged, effective and applicable for a whole range of botanical products. Expansion of multiresidue procedures to include newly registered pesticides is also a challenge as the number of pesticides used globally will continue to increase. The goals of improving methods to become less labor intensive, faster, and high-throughput are also highly desired.

Acknowledgments

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Chapter 24

Detecting the Components of Botanical Mixtures by Single-Strand Conformation Polymorphism Analysis

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Botanical supplements are increasingly being used in the United States, and of major importance is the authenticity of their ingredients. Here we describe a molecular method for species identification that takes advantage of the species variation in the internal-transcribed spacer region of the 18S-23S rDNA of plants. We show that analysis of the ITS-2 region by single-strand conformation polymorphism facilitates the detection of botanical ingredients and potentially contaminating plant material, which can subsequently be identified by DNA sequencing. Our method is useful for authenticating plant species prior to their being processed for manufacture as botanical products and also for recognizing contaminating or adulterating species in dried or powdered botanicals.

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Introduction

Herbal supplements, also known as botanicals, represent a market that has been growing at a rapid pace over the past 15 years. In the United States alone, it is estimated that over 80 million people use some type of botanical supplement, and in 2008, the U.S. market was an estimated $4.8 billion (*1*). In many parts of the world, particularly in developing countries, herbal products are utilized almost exclusively for health care, as pharmaceuticals are either too expensive or not readily available. For example, in rural and urban South Africa, approximately 60-70% of the people use some type of indigenous herbal medicine (*2*).

The medicinal properties of botanicals result from secondary plant products contained within various tissues and organs of the plants. Phytochemicals such as alkaloids, phenylpropanoids, and terpenoids are well documented for their pharmacological activities. However, even though more information is being obtained on the bioactivity of these compounds, the efficacy of the botanical supplements used to deliver them may be questionable due to lack of standardization and defined quality control standards for cultivation and processing of the plant material. Different commercial products have been found to be highly variable in pharmacologically relevant phytochemicals following analysis (*3*, *4*). Such variability could be due to a multitude of factors, including different harvesting and processing techniques used by diverse manufacturers. Moreover, problems also arise because of misidentification of plant material or a confusion of plant names. Hence, a major concern of consumers is the authenticity of the botanical supplements they use. This concern has been exacerbated by actual incidents where the presence of an undesirable botanical resulted in illness. In one case, two women were hospitalized because of digoxin poisoning after taking a herbal product that was contaminated with *Digitalis lanata* (*5*). In another instance, a number of patients in a clinical weight loss trial developed severe nephrotoxicity, in some cases resulting in irreversible damage and the need for kidney transplants (*6*). This toxicity effect was determined to be due to the presence of *Aristolochiza fangchi*, which had been supplied instead of *Stephania tetrandra* (*7*).

Identification and authentication of botanical material used to make herbal products has traditionally relied on morphological characteristics and phytochemical markers (*8*). More recently, molecular methods, such as sequencing, RAPD, RFLP and microarrays have been reported (see (*9*) for review). However, although these methods can be used to confirm the presence of specific species, they are not as readily applied to determining and identifying multiple species in a product. We therefore sought to develop a DNA-based method to identify the plant species present in a given sample of plant material, whether it be present as a single component or a mixture. Such a technique would be useful for identifying the contents of botanical supplements and for confirming the identity of the raw material used for preparing botanical mixtures. DNA sequences give the most unambiguous results because DNA does not change in response to environmental triggers, which might be a factor when using phytochemical marker compounds for analysis. In addition, a large database of DNA sequences cataloging various gene sequences is available, particularly

for the Internal Transcribed Spacer (ITS) region in plants (*10*). We assessed the utility of the two ITS regions, ITS-1 and ITS-2, as well as the chloroplast *matK* gene (*11*) in an analysis of plant material by Single-Strand Conformation Polymorphism (SSCP). We found that the use of the ITS-2 region distinguishes between individual plant species. We further show that SSCP can be used to verify the presence of individual species in a botanical product and to reveal whether contaminating species are present, the identity of which can be subsequently determined by DNA sequencing.

Materials and Methods

DNA Extraction

A modified CTAB procedure (*12*) was used to extract genomic DNA from fresh leaves of alfalfa (*Medicago sativa)*, red clover (*Trifolium pratense)*, woad (*Isatis indigotica)*, European licorice (*Glycyrrhiza glabra)*, Chinese licorice (*Glycyrrhiza uralensis)*, or plant material contained in a commercial red clover product and two different commercial alfalfa or European licorice dietary supplements. Aliquots of DNA were run on a 1% agarose gel to check the quality of the DNA. A repair reaction as described by LeRoy et al. (*13*) was used on those samples that appeared degraded as evidenced by a smear on a gel and failure to produce a PCR product.

PCR Amplification

The polymerase chain reaction was carried out in a final volume of 20 µl with 1U Eppendorf Hotmaster Taq, 1x PCR buffer, 1.5 mM $MgCl_2$, 100 ng of each primer, 1 mM of each deoxynucleotide (dATP, dCTP, dTTP, dGTP) and 1 µl of genomic DNA from fresh tissue or 4 µl of repaired DNA. The primers used, as described in Blattner et al. (*14*), were ITS-A and ITS-C, which amplifies the ITS-1 region between the 18S and 5.8S rRNA genes or the ITS-D and ITS-B, which amplifies the ITS-2 regions between the 5.8S and 28S rRNA genes. The *matK* primers were designed by aligning representative sequences from the database and designing degenerate primers to conserved regions. The forward primer F2 5′TATGSACTTGCTYATRRTCAT3′ and reverse primer R3 5′GAACYAAKATTTCCARAT3′ produce a 400 bp fragment. The reverse primer for ITS-2 or *matK* was phosphorylated at the 5′ end to promote single-strand digestion by lambda exonuclease (*15*). PCR was conducted at 94°C for 5 min, followed by 25 cycles of 30 s 94°C, 30 s 57°C, 90 s at 68°C, followed by a final extension step at 68°C for 10 min.

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Genetic Profiling by Single Strand Conformational Polymorphism (SSCP)

The PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA). Ten µl of the purified product was either heat-denatured or digested by lambda exonuclease (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) at 37°C for 2 hours. The digested product was purified with the Qiagen Minielute kit and eluted in 10 µl 1 M Tris-HCl. Eight µl of denaturing loading buffer (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol) were added to each eluted product and incubated at 95°C for 3 min and snap cooled on ice. The entire sample was loaded on a 0.6X MDE (Mutational Detection Enhancement) gel (Cambrex, Rockland, ME) using 0.4 mm spacers on a Vertical Gel Electrophoresis System (BRL, LifeTech, Inc., MD) and a 1X TBE (*16*) running buffer. Gels were run at room temperature at 7 mA and 90 V for 14 hours. DNA was visualized by soaking the gels in 1 µg/mL ethidium bromide for 30 min.

Extraction, Reamplification, and Sequencing

Bands were cut out of ethidium bromide-stained MDE gels and eluted at 37°C for 2 hours in an elution buffer (0.5 M NH_4OAc, 10 mM MgOAc, 1 mM EDTA, 0.1% SDS). One µl eluent was used in polymerase chain reactions using the ITS-2 primers. The resulting PCR products were run on an agarose gel, purified using the Qiagen gel extraction kit, and directly sequenced using ABI Big Dye Terminator mix and automated sequencing with an ABI 3700 Capillary DNA Analyzer at the UCLA Sequencing Core Facility.

Sequence Analysis

Sequences were compared to the non-redundant database using NCBI BLAST and default settings.

Results

We first confirmed that we could obtain amplifiable DNA from each of our samples. The ITS-2 region could readily be amplified by PCR using DNA extracted from fresh leaves of alfalfa, red clover, European licorice, Chinese licorice, and woad (Figure 1B). Bands showed similar migration, indicating that differentiation based on standard agarose electrophoresis would not distinguish amng the species (Figure 1B). Genomic DNA of the commercial products was found to be degraded when observed on an agarose gel (Figure 1A). Repair reactions on the DNA isolated from the red clover product and first alfalfa product had to be done before a PCR product could be obtained (Figure 1C).

SSCP had previously been successfully applied to the analysis of bacterial and fungal populations, as well as to plants. We desired to extend its application by determining the sensitivity of the method for distinguishing different plant species as well as for detecting the presence of contaminating plant material. We initially looked at five species commonly used in botanicals: alfalfa, red clover,

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European licorice, Chinese licorice, and woad. SSCP analysis of the ITS-2 region for the five different species tested showed that the species could be differentiated based on the migration of single-stranded DNA (Figure 2). In contrast, when we used the ITS-1 region, a distinction between European and Chinese licorice could not be detected (data not shown), similar to the results obtained with the *matK* gene (Figure 3). A comparison of DNA products generated by heat denaturation (Figure 2A) versus single-strand digestion by lambda exonuclease (Figure 2B) showed that exonuclease digestion was more efficient at generating ssDNA products and produced fewer bands in Chinese licorice. Therefore, either method of ssDNA generation was effective at distinguishing between species, but exonuclease digestion was generally better.

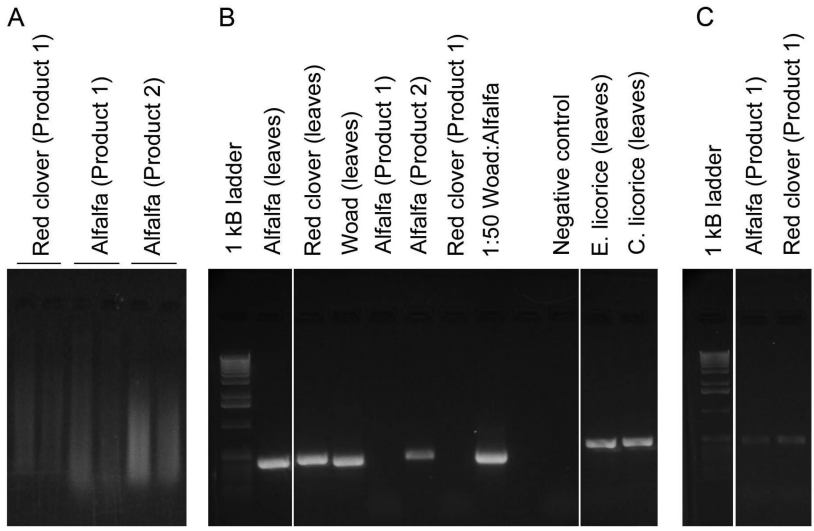

Figure 1. Agarose gel electrophoresis of genomic DNA and the ITS-2 region. (A) Genomic DNA isolated from the contents of commercial alfalfa or red clover capsules was severely degraded as indicted by a smear on an agarose gel. (B) The ITS-2 region was successfully amplified from DNA isolated from fresh leaves and from capsules of the second alfalfa product, but not from the DNA from the first alfalfa product or the red clover product. (C) After a repair reaction, the ITS-2 region could be amplified from DNA from the alfalfa and red clover commercial products.

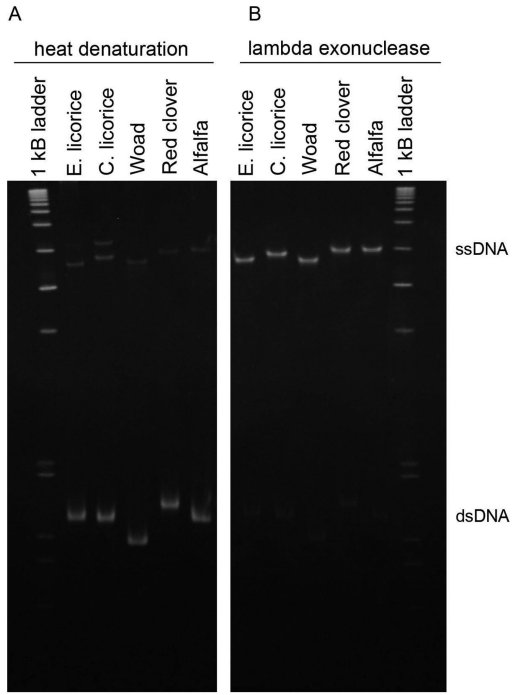

Figure 2. SSCP analysis of the ITS-2 region can be used to distinguish between different plant species. (A) SSCP analysis of PCR products after heat denaturation showed both single-stranded (ssDNA) and double-stranded DNA (dsDNA) products, which illustrated a unique migration pattern for each of the species tested. (B) Lambda exonuclease digestion was more efficient at generating ssDNA.

To verify that SSCP could distinguish species in a mixture, we prepared a simulated plant contamination event by mixing alfalfa and woad genomic DNA. We found that the amplified alfalfa and woad PCR products could be differentiated based on the ssDNA or dsDNA bands generated by PCR amplification of the ITS-2 region and SSCP analysis (Figure 4). To test the sensitivity of this technique for detecting contaminating plant material, we mixed alfalfa DNA with decreasing amounts of woad DNA. The woad contaminant could still be detected at a 1:5000 woad:alfalfa dilution (Figure 4).

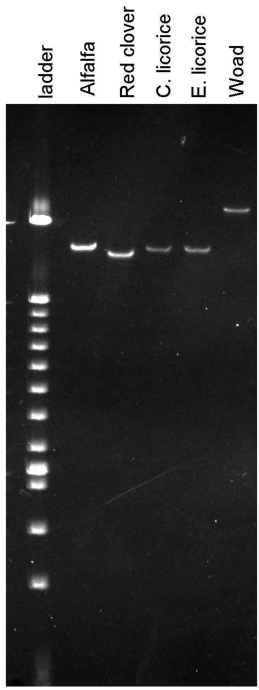

Figure 3. The use of matK in SSCP analysis distinguished alfalfa, red clover, licorice, and woad products. However, no obvious difference in band migration was seen between European licorice and Chinese licorice.

We next applied the SSCP technique to commercial products. We analyzed a red clover product and two different commercial products of alfalfa and European licorice. SSCP analysis of each supplement showed bands corresponding to those expected for each product (Fiure 5). However, the analysis of the commercial supplements also revealed faster or slower migrating bands that might be contaminants (Figure 5). The identity of the single-strand PCR products generated from each of the supplements was determined by extraction of the individual bands, PCR reamplification, and then direct sequencing.

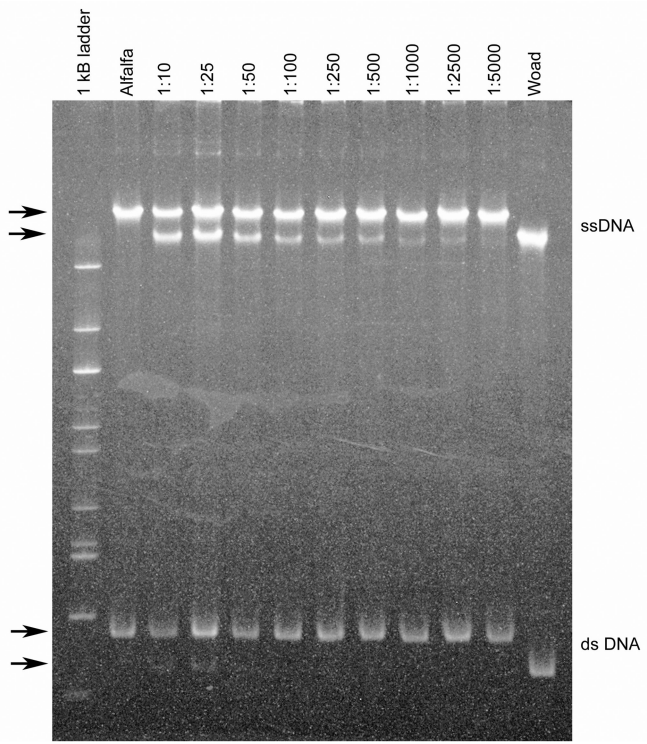

Figure 4. SSCP can detect a woad "contaminant". SSCP analysis of alfalfa containing a simulated woad contaminant using heat denaturation showed that the presence of the two different species could be detected using the ITS-2 region. The woad "contaminant" was detected even when present at a 1:5000 dilution (lower arrow).

The DNA sequence of the bands corresponding to the expected size for the species specified in the product were found to be identical to alfalfa, red clover or European licorice sequences in the database. Sequencing of the bands of the potential contaminants resulted in some ambiguous base calls, suggesting a mixture of species was present. Nevertheless the contaminant in the red clover product was most similar to a *Trifolium* species (92%), albeit one distinct from *T. pratense*. The additional band in the first alfalfa product was found to have greatest similarity to species of *Taraxacum* (dandelion) (93%). The sequence of the contaminant in the second alfalfa supplement was most similar to red clover (95%). The sequences of the contaminants in the European licorice products were most similar to *Cimicifuga racemosa* (black cohosh) (97%) and *Stellaria media* (chickweed) (94%) (data not shown). Cloning and then sequencing the PCR product from the potential contaminating bands rather than direct sequencing would likely result in more robust identifications.

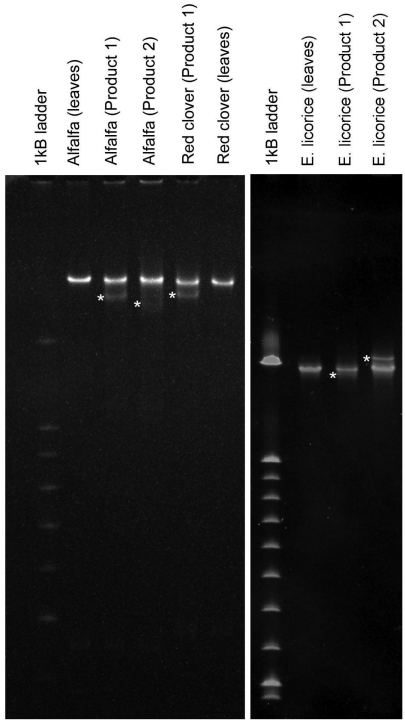

Figure 5. SSCP analysis using lambda exonuclease of commercial alfalfa and red clover products. SSCP analysis of DNA from the commercial alfalfa, red clover, and European licorice products produce a PCR product that migrates to a distance similar to that of DNA from plants of alfalfa, red clover, or European licorice. However, each of the commercial products contains additional faster or slower migrating bands. Asterisks have been placed to the left of these putative contaminants.

Discussion

Molecular approaches for identifying the components of dietary supplements have been described before, but few can readily detect multiple components in the product. PCR-based methods that have been commonly used for botanical authentication are PCR-RFLP, RAPD, direct sequencing, etc. (*9*, *17*). Mihalov et al. (*18*) used PCR combined with sequencing and DNA fingerprinting to identify ginseng in commercial samples. Through direct sequencing of a PCR-generated DNA band, they identified a gene sequence that indicated that the sample contained soybean. However, such an analysis cannot be used when multiple species are present. Another common way to analyze botanicals is RAPD analysis, which relies on comparisons of known molecular fingerprints. However, this technology is not feasible for identifying species for which a profile does not

already exist. We and others (for examples, see (*19–21*)) have used PCR-RFLP to detect contaminating material. In this method, a PCR fragment is digested by a restriction enzyme to give multiple fragments. Its drawback is the number of steps involved and the possibility that adulterants contain the same restriction site as the species of interest.

Single-strand conformation polymorphism analysis was developed for the detection of mutations, specifically in human DNA (*22*, *23*). However, it has become a useful tool in the study of communities and has successfully been used in population biology for the analysis of bacterial, fungal, and sponge populations (*24*, *25*). Although a few examples of SSCP applied to assess plant genotype have been published (*26*, *27*), overall this method has not been widely utilized for plants, especially to species with medicinal properties, except for cinnamon (*28*). Because SSCP detects even small base differences between species, we believe it to be an ideal procedure for detecting and identifying contaminating and/or adulterating species in botanicals.

Here we show that SSCP can be used for discriminating different plant species from one another and for identifying whether a botanical sample or dietary supplement contains multiple species either as contaminants or adulterants. We found that the ITS-2 region worked well for differentiating plant species and also provided better resolution than ITS-1 or *matK*. For example, SSCP analysis of the nuclear ITS-2 region showed distinct variation for each of the five plant species tested, including European and Chinese licorice, both members of the genus *Glycyrrhiza*. Our results analyzing plant mixtures showed that the individual components of the mixture could be distinguished, demonstrating that this method could be used to assess the contents of a botanical product. Our analysis of commercial products using SSCP, with confirmation by sequencing, illustrates that SSCP is an effective technique for confirming species in mixed populations. Moreover, high-throughput methods of capillary SSCP have been developed (*29*), and these could be adapted to analysis of botanicals, although sequence information would be less readily obtainable due to the lack of a standard way of collecting the DNA fragments from the capillary for subsequent sequencing.

The main drawback with our method, or any other DNA-based protocol is the need to have amplifiable DNA. Although we have shown previously, and in this report, that even severely degraded DNA can be repaired and amplified, products that exist solely as plant extracts lack DNA and hence cannot be authenticated or identified using these techniques. However, the SSCP method can be applied to any botanical product that contains intact cellular material and for assessing plant material prior to its processing for botanical products.

We have shown that SSCP combined with sequencing allows for the differentiation and identification of specific species, even those that are very closely related. Therefore, in conjunction with more traditional methods of authentication of botanicals such as microscopic analysis or the assessment of marker compounds, SSCP could be used to demonstrate unequivocally whether contaminants or adulterants are present, and moreover, facilitate their exact identification.

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Editors' Biographies

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Susan E. Ebeler is a professor in the Department of Viticulture and Enology at the University of California, Davis. Her research is focused on development and application of analytical chemistry techniques to study flavor chemistry and the physico-chemical interactions of flavors with nonvolatile wine components. She also studies the chemical mechanisms for the observed health effects of wine components. At UC Davis, she teaches undergraduate and graduate classes on the analysis of grapes and wines as well as the flavor chemistry of foods and beverages.

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Gary Takeoka obtained his Ph.D. in Agricultural and Environmental Chemistry from the University of California, Davis in 1986. In 1987, he was a postdoctoral fellow at the Nestlé Research Center in Vers-chez-les-Blanc, Switzerland. After two additional post-doctoral appointments with Nabisco Brands and Planters LifeSavers, he joined the U.S. Department of Agriculture in Albany, California. He is currently a research chemist in the Processed Foods Research Unit. His research interests include natural products chemistry and flavor chemistry.

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Peter Winterhalter is head of the Institute of Food Chemistry at the Technische Universität Braunschweig (TUBS), Germany. He graduated from the University of Karlsruhe and obtained his Ph.D. from Würzburg University in 1988. In 1989, he was a postdoctoral fellow at the Australian Wine Research Institute, Adelaide, before returning to Würzburg University as a Research Fellow. In 1995, he was appointed as Professor of Food Chemistry at the University of Erlangen-Nürnberg before finally moving to TUBS in 1997 where he was dean of the Faculty of Chemistry and Pharmacy. His research activities focus on natural products/food chemistry and include aroma precursors, countercurrent chromatography, food authentication, natural pigments, and wine research. He has published more than 250 scientific papers and edited 5 books.

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