

Authentication of Food and Wine

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Authentication of Food and Wine

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Foreword

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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 previously published papers are not accepted.

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Preface

The ability to ensure the integrity of our food supply has challenged analytical chemists for hundreds of years. Historically, identifying and confirming adulteration of foods and beverages has been the focus of much scientific research and regulatory action. However, more recently, many commodities and processed foods have sought to gain marketing advantages and consumer acceptance by using claims of specific regional or country-of-origin sourcing and species or varietal-related properties. Many food retailers are now facing voluntary or mandatory labeling requirements that will indicate this origin and species or varietal information. Authentication of these claims remains a significant challenge, however.

This book compiles the latest research on food and beverage authentication using a variety of analytical techniques that can be applied to a range of matrices. The chapters are written by international experts in their respective fields who were invited to participate in a recent American Chemical Society (ACS) symposium on *Authentication of Food and Wine* held in San Diego, California in March 2005.

The first three chapters present an overview and historical perspective on food authentication and some of the current analytical methodologies, ranging from isotope ratio mass spectroscopy, to enantiomeric differentiation, to the use of molecular biology tools such as polymerase chain reaction (PCR) are discussed. In particular, the increasing use of and presence of genetically modified organisms (GMOs) in our food supply presents unique analytical challenges for authenticating food and beverage ingredients.

The essential oil industry has long been required to confirm the authenticity of its ingredients—including confirmation of source (e.g., plant species or variety) and whether the components are “naturally-derived” or “chemically” synthesized. Chapters 4–6 focus on these topics.

The application of sophisticated analytical tools for the authentication of food and beverage matrices are then highlighted in

Chapters 7–10. Foods, such as olive oil and seafood, and beverages, such as tea and fruit juice, are emphasized. Alcoholic beverages, including wine and distilled spirits, have a long history of labeling and marketing with respect to region of production, grape variety or sugar source, and age. However, the analytical tools to authenticate these claims have been limited. Chapters 11–18 focus on recent research in this area.

Finally, authentication of the source and composition of herbs, herbal products, and botanicals used as dietary supplements is a growing concern. Chapters 19 and 20 highlight recent research that uses both chemical and molecular approaches to identify the active components as well as the potential contaminants in a range of herbal medicinal materials.

While each food, beverage, or botanical matrix presents unique challenges, all research in this area requires the availability of verifiable standard materials and, in most cases, more than one analytical tool will be required to confirm product authenticity (e.g., combinations of molecular and chemical approaches or combinations of two or more chemical analyses). In addition, in many cases, sophisticated statistical approaches will be required to evaluate large and complex data sets. As a result, a multidisciplinary approach will be critical to our future ability to authenticate foods and beverages not only to confirm adulteration but also to confirm geographic source, age, or variety claims. This book is a reflection of this exciting mixture of multidisciplinary topics and we hope that it will be a valuable resource for research, industrial, and regulatory scientists who are engaged in food and beverage authentication in the food, pharmaceutical, and nutraceutical industries.

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Chapter 1

Authentication of Food and Wine

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Consumers increasingly demand reassurance of the origin and content of their food. The process by which a food is verified as complying with its label description is called food authentication. In order to ensure authenticity various elaborate testing protocols have evolved, and new methods continue to be developed to match increasingly sophisticated adulteration schemes. This introductory chapter will give a brief overview on the history of food adulteration as well as the methods used for the assessment of food authenticity. Finally, examples of our own research in this field will be presented.

Introduction

The adulteration of foods and beverages has been a problem ever since we have stopped producing our own food and had to rely on commercial supplies. There have always been (and will always be) some unscrupulous suppliers who are prepared to maximize their profits by illegal practices. In many cases this comprises the addition of a safe but lesser valued material to a higher-priced product. As such, the danger to the public is relatively small. However there are examples of fraud that have seriously threatened human health, such as the toxic oil syndrome (TOS), a devastating disease that occurred in Spain in 1981. TOS was associated with the consumption of aniline-denatured and refined rapeseed oil that had been sold as olive oil. 400 people died and until today, 25 years after the poisoning event, TOS survivors still suffer symptoms. Wine is another commodity

of wholesale merchants was founded in London in 1180 and was later incorporated into a spicers' guild. In addition to spices, also bread, as one of the staples of life, came under legal control. Short weights brought corporal punishment such as immersion in the pond and for repeated offenses, confiscation of the bakery. Punishment during the Middle Ages was rigorous and often related to the crime. In Biebrich (Germany) in 1482 a vintner who had adulterated his product was condemned to drink six quarts of his own wine, from which he died. In the city of Nuremberg in 1444 a falsifier of saffron was burnt over a fire of his own saffron, others were buried alive in the spice.

The sixteenth and seventeenth centuries offered new opportunities to adulterers, with the import of tea, coffee, chocolate, and sugar from the new world. The cruder forms of adulteration were replaced by substitutions of a more skillful and novel character, beyond the scope of the revenue of the food inspectors of the time to detect. Luxuries such as tea were adulterated twice, first in China and furthermore by the home merchants. The importers added iron filings, clay and gypsum to increase weight. Also foreign leaves and exhausted tea leaves were routinely used as stretchers. Coffee had a somewhat similar history to that of tea.

With the onset of the industrial revolution, thousands of people began to move to the new centers of industrialisation. The nourishing of these ancient peasants changed fundamentally; all of a sudden, they were totally dependent on foods produced and sold by others. Analytical chemistry was still in the process of development. On this basis, and especially in the absence of any legal or administrative obstacles, it is not surprising that the adulteration of foods flourished. Especially heavy metals and other inorganic elements turned out to be cheap and suitable to restore e.g. the color of a skimmed milk and other adulterated foodstuffs. In London, the coloring of milk was so common that housewives refused to buy the uncolored product, thinking it was adulterated.

Frederick Accum, a German chemist and one of the pioneers in food authentication, published a first report on food adulteration in Britain in 1820 (4). However, it took another forty years and another series of food-poisoning incidents until the first "Adulteration of Food and Drink Act" was passed in Britain in 1860. The legislative history of other countries went through similar cycles, and most countries passed their first food laws in the second half of the 19th century. Although, in contrast to today, these first laws followed the principle of a negative listing (i.e. substances not allowed for use), they were already driven by the main principles of today's food regulations all over the world: the protection of the consumer from health risks and from fraud. With regard to the worldwide development of food legislation in the 20th century as well as the attempted harmonization through FAO/WHO activities in food control, the interested reader is referred to Ref. (5).

that was frequently the subject of fraud. Whereas in the Middle Ages lead acetate (“lead sugar”) was used as a sweetener of wine and cider, some Austrian winemakers in 1985 added ethylene glycol to wine to upgrade it to table wine quality. As a result the Austrian wine export in the following years almost broke down.

There are ample opportunities for the adulteration of food and almost each year a new scandal hits the headline. According to the Food, Drug and Cosmetic Act (21 U.S.C. 342), a food is deemed adulterated if:

- any valuable constituent has been, in whole or in part, omitted;
- any substance has been substituted wholly or in part thereof;
- damage or inferiority has been concealed in any manner; or
- any substance has been added thereto or mixed therewith so as to increase its bulk weight or reduce its quality or strength, or make it appear better or of greater value than it is.

Most recently, the illegal dyes Sudan I and Para Red were detected in Worcester sauce and caused the recall of more than 600 food brands in the UK (1). This clearly demonstrates that despite all efforts in authenticity testing by the authorities, the desire of certain people to make a fraudulent profit will always exist.

History of Food Adulteration/Authentication

Evidence from the earliest historical writings indicates that governing authorities were already then concerned with codifying rules to protect consumers from dishonest practices in the sale of food. Assyrian tablets described the method to be used in determining the correct weights and measures for grains, and Egyptian scrolls prescribed the labelling to be applied to certain food. In ancient Athens, beer and wines were inspected for purity and soundness, and the Romans had a well organized state food system to protect consumers from fraud or spoiled produce. An extensive review on the history of the adulteration of food has been published by F.L. Hart (2). Only some aspects of this work can be mentioned here. Initially possibilities for adulteration were limited because food – at that time flours and meals - were almost always made at home. This situation changed with urbanisation, when trade emerged. Honey, wines, ales, and oils were the first candidates for adulteration. Pliny the Elder had one book dealing specifically with wine which was frequently diluted with water or colored and flavored with noxious herbs (3). By the thirteenth century, commerce in Europe was controlled by voluntary mercantile associations or “guilds” with their own codes and regulations in order to maintain the workmanship and quality of their wares. A pepperers' guild

Methods for Food Authentication

Initial methods for the detection of fraud were limited and rather simple. Honey was examined by duly appointed honey inspectors and the so-called “ale-tasters” (in England) or “Bierkiesers” (in Germany) were responsible for the quality of ale and beer. They checked not only the taste of the beverage but also its malt content using a simple testing protocol. The German version of this authenticity test was as follows: some beer was poured on a wooden bench. Three testers with leather breeches were sitting on the wet spot for a certain time. Then the testers got up and if the bench adhered to the leather breeches, the malt content of the beer was acceptable. In this context, it should also be mentioned that one of the oldest food laws, i.e. the German purity law for beer dates back to the year 1516 and is still valid today in Germany.

In the 19th century new developments such as the analytical balance as well as the microscope enabled analysts to more easily detect foreign substances in foods (6). Today analytical methods for detection of fraud are certainly much more advanced. Modern techniques used in food authentication combine on the one hand chromatographic and spectroscopic methods with chemometrics for compositional analyses, and on the other hand electrophoretic, immunochemical, as well as DNA methods for species and GMO identification. Authenticity issues nowadays also include the determination of the geographical origin of a certain product which is a particularly challenging task. In this regard stable isotope analyses and the use of multi-element data are the most promising approaches.

Since many details of authenticity testing of different foods and beverages are presented in the following book chapters as well as in the recent literature (7,8), only one example of increasingly sophisticated adulteration methods will be discussed here: the analysis of natural flavor compounds (9).

A breakthrough in the authenticity testing of natural flavors and essential oils was the introduction of enantioselective capillary gas chromatography (enantio cGC) approximately two decades ago which allowed the determination of enantiomeric ratios of chiral flavor compounds. In those cases where racemic mixtures were detected, the addition of synthetic flavorants was most likely. For a couple of years this approach worked well, however, with the advent of innovative preparative techniques (e.g. simulated moving bed chromatography) it was possible to separate synthetic racemates on a large scale and the addition of enantiomerically pure compounds was at least technically feasible. Hence, enantioselective cGC analysis was no longer sufficient for an unambiguous authenticity assessment, it had to be combined with isotope ratio mass spectrometry (IRMS) using a pyrolysis interface. This method added information concerning the overall $\delta^2\text{H}$ - and $\delta^{13}\text{C}$ isotopic values of the molecule and helped to distinguish between natural and synthetic flavor compounds provided that suitable data bases were available. But again it seems that some adulterators found

ways to overcome this elaborate testing protocol. Remaud et al. (10) demonstrated the presence of isotopically enriched vanillin that was obviously obtained by synthesis in the intention to overcome the IRMS-based authenticity test. For such samples the ultimate weapon in authenticity testing has to be applied, the so-called site-specific natural isotope fractionation (SNIF) NMR, a trademark of the Eurofins company. With the SNIF NMR method that is still restricted to major compounds of a flavor extract (e.g. vanillin, benzaldehyde), the site-specific distribution of the ^2H nucleus in a molecule - which is caused by isotope discrimination during biosynthesis - is measured. This distribution was found to be typical for a particular aroma compound of a defined source.

This example clearly demonstrates the need for innovative analytical methods and continued vigilance in the determination of food authenticity. The main lessons from the continuing problems with food adulteration is that nothing can ever be taken for granted. Disreputable suppliers will always be tempted to "improve" quality by the addition of some illegal or inappropriate substances. In view of this, the food authorities and food analysts must always be alert to ensure the quality and safety of our food.

Examples

Example 1: Differentiation between Arabica and Robusta Coffees

Of major importance for the world coffee production are the two species *coffea arabica* and *coffea canephora* var. *robusta*. These two economically important species differ especially in flavor and, because of that, in price, too. A unit of high quality arabica costs nearly twice as much as robusta coffee (Table 1). Often the high-quality arabicas are mixed with less expensive robustas. Therefore it is very important to have a possibility for controlling the quality of the products with appropriate methods for the discrimination of the two species.

Robusta beans are smaller in size and rounder in shape than arabica beans. Hence, a differentiation by comparison of the bean-size before roasting and grinding is possible. Also quantities over 15 % of robusta in arabica blends can be detected by sensory testing. But the most important method for discrimination is an analysis of the ingredients. Latest research has described the discrimination between the two species on the basis of amino acid enantiomers (11). Another very useful analytical parameter is the content of the diterpene 16-O-methylcafestol (16-OMC), which is only present in robusta coffee in a concentration range of 0.8 – 1.4 g/kg (12). Therefore an official method for the determination of 16-OMC in roasted coffee has been developed, employing reversed phase high-performance liquid chromatography (13). This method allows detection of up to 2 % of robusta in arabica blends. When we started our investigation, there was worldwide no 16-OMC as reference standard commercially available. The lack of an authentic reference

prompted us to develop an efficient method for the preparative isolation of 16-OMC using the all-liquid chromatography technique of countercurrent chromatography (CCC).

Table 1. Comparison Arabica vs. Robusta Coffees

	ARABICA	ROBUSTA
World production	75 %	25 %
Cultivation latitude	23° N – 25° S	10° N – 10° S
Altitude	600 – 2200 m	0 – 800 m
Temperature [°C]	15 – 24	18 – 36
Rain [mm/year]	1200 – 2200	2200 – 3000
Ripening [months]	7 – 9	9 – 11
Flavour	mild acid, good body	strong aroma (e.g. 2-methylisoborneol)
Costs	~ 65 US-cts/lb	~ 38 US-cts/lb

CCC is an automated version of liquid-liquid extraction, comparable to the repeated partitioning of an analyte between two immiscible phases by vigorous mixing in a separatory funnel. In high speed CCC (HSCCC) the separation takes place in a so-called “multi-layer coil” that is made by wrapping inert Teflon tubing around a holder in multiple layers. The tubing usually has an inner diameter between 1.6 and 2.6 mm and the length can reach 160 m. Multiple coils can be connected in series to increase the total volume of the instrument and the sample capacity. During separation the coil is rotated in a planetary fashion; it rotates at 800 to 1000 rpm around its own ‘planetary’ axis and simultaneously around a parallel ‘solar’ axis. This planetary rotation has two effects: The rotation creates a fluctuating acceleration field which enables vigorous mixing of the two phases followed by settling within the coil. In areas of the coil which are close to the centre of rotation, the force field is weak. As a consequence the phases are mixed. At a further point of their orbit, when they are far away from the centre of rotation, the force field becomes stronger and the two phases are separated. Alternate mixing and settling is repeated with each rotation and in this way up to 50,000 partitioning steps per hour can be achieved. Today the technique is routinely applied for the fractionation of complex mixtures and the isolation of reference compounds. It is gentle, inexpensive and versatile (14,15). Examples for successful purification of numerous compounds have been published (16). As an example the HSCCC separation of 16-OMC is shown in Figure 1.

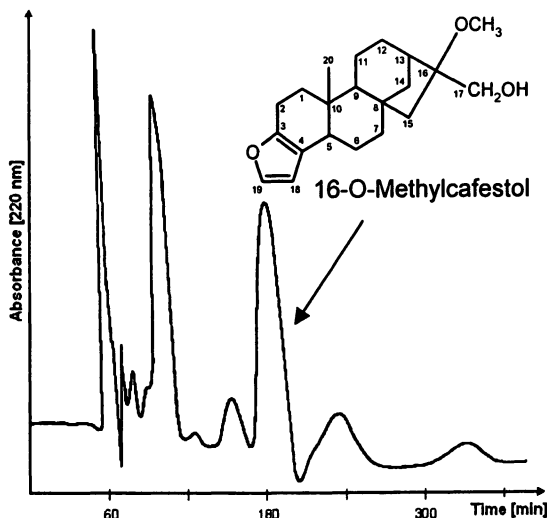


Figure 1. HSCCC-separation of 16-O-methylcafestol (16-OMC). Solvent system hexane/ethyl acetate/ ethanol/water (5/2/5/2 v/v/v/v, less dense layer as stationary phase, flow rate: 4.0 mL/min).

For the isolation of 16-OMC, roasted robusta coffee beans (India) were ground and extracted with *tert*-butyl methyl ether (TBME). The coffee lipids were saponified under reflux with ethanolic potassium hydroxide and the unsaponifiable matter was separated by liquid-liquid extraction using TBME. After removal of the solvent, the residue was separated by HSCCC (cf. Figure 1). One gram of the unsaponifiable matter of robusta beans could be applied for each HSCCC separation and 140 mg of pure 16-OMC was obtained in each run (17).

Example 2: Authentication of Wine Age

Wine labels often indicate the year of vintage and may also give some additional information on the quality, such as e. g. Reserva or Grand Reserva. In the case of Spanish wines Reserva means that the wine is at least three years old (one year storage in casks plus two additional years in the bottle). Gran Reserva wines even require five years of storage (two years in cask plus 3 years in bottle). Since prolonged storage adds costs to the production process the question arises whether or not the requirements for Reserva wines can be monitored by analytical methods. One of the oldest methods for determining the age of a wine is carbon-14 dating that takes advantages of the natural decay of the ¹⁴C isotope content in

ethanol. Several factors can effect the results and only for very old wine does the method have some relevance. For rather young wines alternative methods are required. Various attempts have been made to find chemical markers that may give a hint on the age of a wine but most of them failed, such as e. g. vitisin A (18). Recently we were able to isolate and structurally identify a reaction product of malvidin 3-*O*- β -D-glucoside and 4-vinylcatechol in Pinotage wines, which was named pinotin A (19). Thorough investigations on the pathway of formation revealed that pinotin A is generated by a direct reaction between malvidin 3-glucoside and caffeic acid (Figure 2) (20).

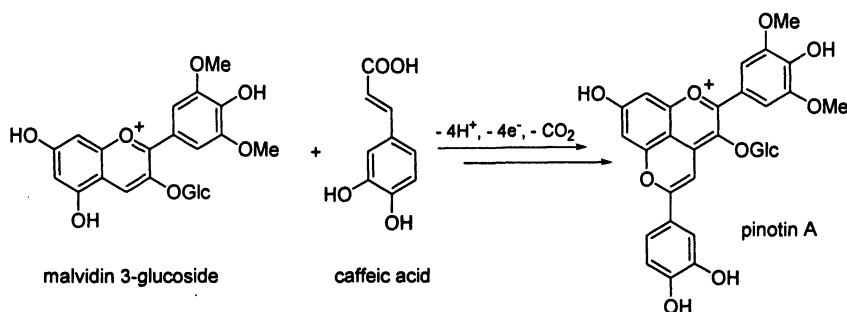


Figure 2. Formation of pinotin A by reaction of malvidin-3-glucoside with caffeic acid (20).

Similarly, we could show that the major route to the related 4-vinylphenol, 4-guaiacol, and 4-vinylsyringol adducts is through a direct reaction of malvidin 3-glucoside with coumaric acid, ferulic acid, and sinapic acid, respectively. The purely chemical reaction pathway leading to pinotin A and its high pH-stability make the pigment potentially attractive as a possible ageing indicator for red wines.

Pinotin A concentration increased with wine age (Figure 3). Various factors that influence the formation of pinotin A in Pinotage red wines have been studied. Apart from wine age, the influence of the concentrations of the direct precursors caffeic acid and malvidin 3-glucoside as well as of the structurally related caftaric acid was examined. Details of this study have been published elsewhere (21,22). Whereas a linear regression model using age and malvidin 3-glucoside provided a relatively poor fit ($R^2 = 39\%$), a model using age and caffeic acid worked much better ($R^2 = 75\%$).

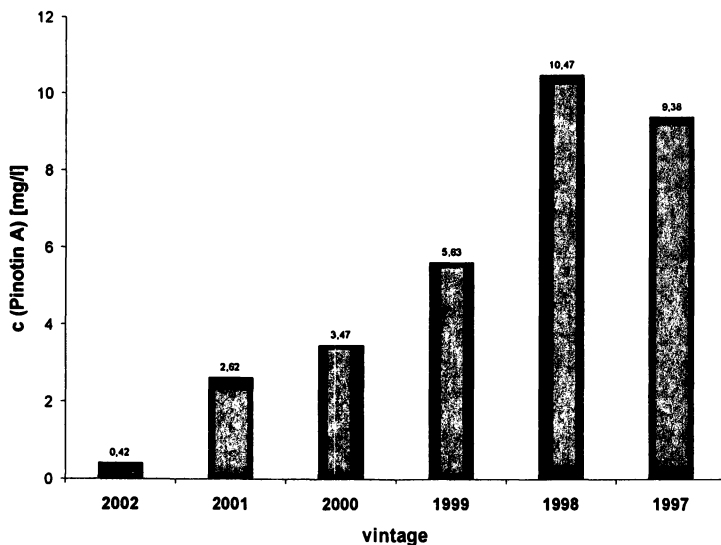


Figure 3. Pinotin A concentration in Pinotage wines of different age.

For quantitative prediction of the pinotin A concentration (in μmol) the following equation can be used

$$\log(\text{pinotin A}) = -4.07 + 0.54 \times \text{age} + 0.84 \times \log(\text{caffeic acid}),$$

or the retransformed version:

$$\text{pinotin A (in } \mu\text{mol)} = \exp(-4.07 + 0.54 \times \text{age}) \times (\text{caffeic acid})^{0.84}.$$

Our data clearly indicated that pinotin A formation depends to a larger extent on the concentration of caffeic acid than on malvidin 3-glucoside. The high amounts of malvidin 3-glucoside present in young wines are rapidly degraded by various side reactions and only a very small percentage was converted into pinotin A. The superproportional formation of pinotin A commences when interfering reactions become less likely due to a lower concentration of the reactants, while the caffeic acid level remains rather stable throughout the ageing process. A minimum concentration of 5-10 mg/L malvidin 3-glucoside was required to maintain a reaction rate high enough to compensate for the simultaneous incorporation of pinotin A into polymeric pigments. The pinotin A content can increase until the wines are approximately 5 to 6 years old. Pinotin A is therefore a potential chemical marker for wine age, but an exact prediction of the vintage still requires a higher number of data sets.

Pinotage red wines were the ideal object for this study on wine age as they exhibit unusually high concentrations of caffeic acid. Nonetheless, the formation of pinotin A is not limited to this cultivar and will proceed in all wines containing free caffeic acid. In wines with higher amounts of other hydroxycinnamic acids, structurally similar pyranoanthocyanins may form following the same reaction mechanism. The content of the resulting ageing products in wines from other varieties is the subject of continuous research.

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Chapter 2

Detection and Quantification of Roundup Ready Soy in Food Samples using Conventional and Real-Time Polymerase Chain Reaction

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Transgenic soybean line GTS-40-3-2, marketed under the trade name Roundup Ready® (RR) Soy was first approved in North America for environmental release, feed products and food products in 1994 and has since been approved in many other countries around the world. Consumer concern issues with regards to genetically modified plants and plant products have resulted in mandatory labeling requirements in several countries for foods derived from genetically engineered crops. One requirement for labeling is the ability to detect and accurately quantify the amount of transgenic material present in foods. Two assays were evaluated. A conventional qualitative PCR assay to detect the presence of soy and RR soy and a real-time PCR to quantify the amount of RR soy present in samples that tested positive in the first assay. PCR controls consisted of certified RR soy reference material, single transgenic soybeans and a processed food sample containing a known amount of RR soy. To test real-world applicability, a number of common grocery store food items that contain soy based products were tested. For some samples, significant differences in amplification efficiencies during the quantitative PCR assays were observed compared to the controls, resulting in potentially large errors in quantification.

Introduction

In 2003, there was approximately 672 million acres of arable land under cultivation worldwide. Of this, about 25% was used to grow genetically modified (GM) crops, for a total of over 162 million acres. This percentage was higher in 2004 (over 200 million acres) and will likely increase further, especially in countries like Brazil and China. The United States is the largest producer with over 100 million acres of land used to grow GM crops. The other major producers include Argentina (34 million acres), Canada (10 million acres), Brazil (8.4 million acres), China (7 million acres), and South Africa (1 million acres). Together, these 6 countries account for approximately 99% of global GM production. The European Union (EU) has had regulations for the labeling of novel foods since 1997. EU regulations stipulate that products containing an ingredient of which 0.9% or more originates from a GM product, must be labeled. Other countries such as Japan, Korea, Indonesia, Australia and New Zealand have also adopted mandatory labeling requirements, whereas countries like Canada, Argentina and the USA have voluntary labeling schemes.

Since the first GM products were approved in the early to mid-1990's, over 60 plant products containing unique transgenic DNA events have been approved for food (8). Herbicide tolerant (GTS-40-3-2) soybean (9), first approved in 1994 in the USA, is one of the most widely grown in Canada where it was approved in 1995. By 2002, over 100 varieties containing this event have been registered in Canada. In 2002 over 75% of the soybean seed stock in USA was GTS-40-3-2, in Canada, 30% and Brazil 20%. These 3 countries account for over 45% of the total global production of soybean and over 50% of worldwide soybean exports. As a result over 30% of world soybean export is GTS-40-3-2. GTS-40-3-2, also known as Roundup Ready® (RR), was developed by Monsanto and confers tolerance to the glyphosate based herbicide Roundup®. The GTS-40-3-2 event consists of an enhanced cauliflower mosaic virus 35S promoter, a CTP4 leader sequence from *Petunia hybrida*, the 5-enol-pyruvylshikimate-3-phosphate synthase (EPSPS) gene conferring the herbicide tolerance followed by an *Agrobacterium tumefaciens* nopaline synthase terminator (9,10). A number of methods have been developed for the detection of RR soy. These include protein based methods for the detection of the EPSPS gene product in transgenic raw or unprocessed soy products (9,11,12,13) and PCR based methods, both qualitative and quantitative which can also be used for the more highly processed soy based food products (1,3,4,5,6,7,9,11,14,15,16). Several of these methods have been officially approved or validated by other government agencies (1,9,12,17). Considering the large amount of RR soy on the world market, and the regulatory requirements of many countries concerning GM crops, it was decided that GM soyfoods would make a good test study to determine the

feasibility of detecting and quantifying the presence of RR soy in processed food samples. Food samples available through local Canadian supermarkets were analyzed for the presence of Roundup Ready® (RR) DNA using two different PCR protocols to first establish that soy DNA and Roundup Ready® (RR) DNA could be detected. These results were used to help determine from what type of foods it was possible to amplify soy DNA, and to get an initial impression on the prevalence of genetically modified soy in common food products. Those products testing positive were then further analyzed by real-time quantitative PCR, to determine the level of Roundup Ready® soy in these foods. Few studies to date have attempted to quantify the amount of transgenic material in processed foods to determine the practical feasibility of such testing.

Materials and Methods

Soy Food Samples

A selection of 39 soy food products including vegetarian foods, dry foods, snack foods, condiments, desserts, soups, baby formulas, and beverages represented by arrows from high to low after processing (Table I), were chosen from local supermarkets.

Soybean Reference Material

As reference material for qualitative and quantitative analysis, Roundup Ready® soybean (variety: S14M7), was obtained from a Canadian seed distributor. Since the purity of the seed lot was unknown, single seed extractions were done to confirm that individual seeds were transgenic. One of these DNA extractions was then used as reference material. The single seed extract was diluted to 40 ng/μL and serially diluted to 12 pg/μL. Six of these dilutions (5 μL per reaction) were chosen to represent the standard curve encompassing 200 ng - 60 pg of DNA.

Quantitative PCR control. Soybean powder certified reference material (CRM) IRRM-410S (Fluka) 5% Roundup Ready® soy used for quantitative real-time PCR was purchased from Sigma (Canada). A processed soy food sample, meat pâté, from the Genetically Modified Material Analysis (GeMMA), Scheme, Report No. GeM18, with an assigned RR soy content of 8.5%, was also used as a control.

Table I Soy Foods

Food	Soy Ingredient	Food	Soy Ingredient
Miso	fermented beans	Boiled soybean	whole bean
Soy sauce	fermented beans	Dried soybean	whole bean
Natto	fermented beans	Roasted soy nuts	whole bean
TVP	textured soy pro	Soy nut snack	whole bean
Meat alt. 1	textured soy pro	Soy nut spread	whole bean
Meat alt. 2	textured soy pro	Sim. bacon bits	flour/soy protein/hyd. soy pro.
Cheesies	hyd. soy pro.	Protein bar 2	flour/lecithin/iso. soy pro.
Cracker 1	hyd. soy pro.	Biscuit	Flour
Gravy mix 1	oil / hyd. soy pro	Gravy mix 2	flour/ hyd. soy pro.
Frozen dess.	soy pow/iso. soy pro	Bread 1	flour/iso. soy pro./lecithin
Soup	soy milk	Bread 2	defatted flour
Soybean pate	protein	Soup mix	soy powder
Protein bar 1	roasted soybean / iso. soy pro.	Nutritional bar	flour/ iso. soy pro / soybean / lecithin / soy butter
Soy beverage	soy milk	Cheese	flour/oil
Meal replac. bev.	protein	Crispbread	defatted flour
Coffee whitener	whole bean	Cracker 2	flour/lecithin
Yogurt	soy milk	Flour	Flour
Chocolate pud.	soy milk		
Meat alt. 3	soy pro. conc./ iso. soy pro.		
Tofu	soybean curd		
Infant formula 1	oil/ iso. soy pro./soy milk		
Infant formula 2	oil/ iso. soy pro./soy milk		

^bisp: isolated soy protein, ^bTVP: textured vegetable protein, ^ctsp: textured soy protein, ^ahsp: hydrolyzed soy protein, ^espc: soy protein concentrate. (Reproduced with permission from reference 30. Copyright 2004 American Chemical Society.)

Extraction of Genomic DNA

Each soy food product was homogenized in a Waring electric blender, then ground using an ice-cold mortar and pestle. Approximately 200 mg or 1 g samples from the ground material was used in the subsequent DNA extraction procedure. The single S14M7 soybean seed reference material (~200 mg) was ground using a chilled mortar and pestle, while 50 mg of the CRM soybean powder was extracted directly.

Modified Wizard

Briefly, 860 μL of extraction buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% (w/v) sodium dodecyl sulfate), 100 μL (5 M) guanidine hydrochloride and 40 μL (20 mg/mL) proteinase K was added to 200 mg of each ground sample and vortexed to mix thoroughly. For 1 g samples, the extraction buffer, guanidine hydrochloride, and proteinase K were scaled up proportionally. The samples were incubated at 60°C for 30 - 60 min followed by a further incubation at 75°C for 20 min to inactivate the proteinase K. The supernatant generated after centrifugation in an Eppendorf microfuge for 20 min at 16,000 \times g (to pellet debris), was incubated for 10 min at 60°C with RNase A added to a final concentration of 1 mg/mL. Following a 3 min centrifugation at full speed, 1 mL of the supernatant was mixed with 1 mL of WizardTM resin (Promega), then applied to a WizardTM mini column using a 3 or 5 mL capacity disposable Luer-Lok syringe. For samples that had a dark coloured supernatant (roasted soy nuts, biscuit, natto, and simulated bacon bits), the column was washed with 1 mL CQW wash buffer (Nucleospin Food Kit, Macherey-Nagel). All samples were washed with 2 mL 80% ethanol, then centrifuged at full speed for 10 min followed by a 10 min incubation at 37°C, to evaporate residual ethanol. The nucleic acids were eluted in 100 μL of 70°C 10 mM Tris pH 8.0.

Qiagen Stool Kit

The soy sauce, chocolate pudding, and miso homogenized samples were dried at 37°C for 24 h to remove excess moisture. To 1 g of dried sample, ASL buffer (supplied with Qiagen Stool Kit) was added until a consistency was reached such that the sample flowed freely in the tube (2-3 mL). The samples were vortexed until thoroughly homogenized. Following a 5 min incubation at 70°C, the samples were centrifuged at 16,000 \times g for 10 min to remove particulates. The supernatant was divided into multiple 2.0 mL microfuge tubes and 1 InhibitEX tablet added to each (1 tablet / 1.2 mL supernatant). If the volume was less than 1.2 mL per tube additional ASL buffer was added. The

samples were vortexed to suspend the InhibitEX tablet then incubated at room temperature for 1 minute. The samples were centrifuged at full speed for 3 min to pellet the InhibitEX particles. The supernatants were pooled then incubated for 10 min at 60°C with 1 mg/mL final concentration of RNase A. Following centrifugation at 16,000 x g for 3 min, the pooled supernatants were treated with Qiagen Stool Kit, Proteinase K solution and AL (supplied with Qiagen Stool Kit) buffer proportionally (for every 200 μ L of supernatant, 15 μ L and 200 μ L of Proteinase K and AL buffer were added, respectively) according to the manufacturer's instructions. After incubated at 70°C for 10 min the amount of 95% ethanol was added according to manufacturer's instructions. This entire mixture was then applied to the QIAamp spin column. The column was then washed and the DNA was eluted with 100 μ L AE buffer according to manufacturer's instructions.

Determination of Yield and Quality of Nucleic Acid Extracts.

The nucleic acid extracts were measured using the Biochrom Ultraspec 2100 pro spectrophotometer in 200 mM NaOH (9). A conversion factor of 1 OD = 37 ng/ μ L was used to convert absorbance into concentration units. Sample purity was determined by measuring the A_{260}/A_{280} ratio. The extracts (100 ng-200 ng) were further analyzed by electrophoresis on a 0.8% agarose gel containing 0.1 μ g/mL ethidium bromide.

Qualitative PCR Detection of Lectin and Roundup Ready® (RR) Sequences

PCR amplification was performed using 100 - 200 ng of DNA and 100 - 400 ng of DNA for the lectin and RR assays, respectively. Of the extracts that could not be accurately measured spectrophotometrically (cheesies and miso; $A_{260} < 0.1$) 10 μ L of extract was used in the PCR reactions. A 25 μ L reaction contained 1X PCR Buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), $MgCl_2$ (2.8 mM lectin; 1.8 mM RR; 2.0 mM chloroplast tRNA), 0.2 mM dNTP, 0.5 μ M of each primer and 1.25 U Taq polymerase. Sequences of primers (Qiagen) used are shown in Table II. The GeneAmp 9700 (Applied Biosystems) thermocycler was used with the following cycling program for lectin; 94°C for 3 min, 35 cycles of 94°C for 15 s, 60°C for 15 s, 72°C for 20 s, and a final extension at 72°C for 5 min. The program for the RR PCR was similar except that 40 cycles were used. Products were electrophoresed on a 2% (w/v) agarose gel containing 0.1 μ g/mL ethidium bromide.

The limit of detection (LOD) for both the lectin and RR qualitative PCR was determined from serial dilutions of S14M7 soybean seed DNA extracted using the modified Wizard™ method. Dilutions ranging from 50 ng - 10 pg (lectin) and 80 pg - 1 pg (RR) were tested in triplicate. The limit of detection

was defined as the lowest dilution for which the PCR product of expected size was visible by agarose gel electrophoresis in all three replicates. The conversion of mass to genome copy number was based on the haploid genome mass (1C value) for *Glycine max* which is 1.13 pg, obtained from the Plant DNA C-values Database (20).

Real-time Quantitative PCR of the Lectin and Roundup Ready® (RR) gene

Fluorescence resonance energy transfer (FRET) hybridization probes were used to quantify in the LightCycler (Roche) real-time PCR system. The primers and probes (IT Biochem) used for the lectin and RR assays are referenced in Table II, and the cycling conditions are described by Dahinden *et. al* (19). The donor probes for both targets were labeled with fluorescein-5-isothiocyanate (FITC) and the acceptor probes for lectin and RR genes were labeled with R705 and R640, respectively. Sequences of primers and probes used are shown in Table II.

Table II Primers

Primer/Probe	Sequence 5' to 3'	Product Size	Ref.
Lectin Qualitative			
GMO3(forward)	gccctctactccacccccatcc	118 bp	(9)
GMO4(reverse)	gcccatctgcaagccttttgtg		
RR Qualitative			
RR04(forward)	ccccaagttcctaaatcttcaagt	180 bp	(18)
RR05(reverse)	tgcgggccggctgcttgca		
Lectin Quantitative			
LecLC-F(forward)	cctctactccacccccatcca	114 bp	(19)
LecLC-R(reverse)	ccatctgcaagccttttgtg		
LecLCFITC-1 (probe)	ttgccagcttcgccgttc-FITC		
LecLCR705-1 (probe)	Red705-ttcaacttcaccttctatgccctgac		
RR Quantitative			
RRLC-F20(forward)	accgtcttcccgttaccttg	119 bp	(19)
RRLC-R15(reverse)	gccggcggtgtgag		
RRSoyaLCFITC-1 (probe)	gccgatggcctccgcaca-FITC		
RRSoyaLCR640-1 (probe)	Red640-gaagtcggcgtgctgctcg		

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PCR was performed in glass capillary tubes supplied by Roche. A total reaction volume of 20 μL contained 5 μL (15 - 200 ng) DNA template, 50 mM Tris pH 8.3, 0.4 μM of each probe, 0.25 mM of each dNTP, 0.5 μM of each primer, 250 $\mu\text{g}/\mu\text{L}$ BSA and 1 Unit of Fast Start Taq Polymerase (Roche). The lectin reactions contained 4.0 mM MgCl_2 and the RR reactions contained 2.5 mM MgCl_2 . A serial dilution of over 3×10^3 (200 ng to 60 pg per reaction) of total genomic DNA extracted from the S14M7 single seed, amplified in separate reaction tubes using either the lectin primers/probes or RR primers/probes, was used to generate the calibration curves for lectin and RR, respectively. The standard curves were the regression of crossing point (Cp) versus log of the ng of standard in each reaction, where the crossing point is the cycle at which the reaction fluorescence increases above a baseline level defined by the software (see Figure 1, Table III).

Each food sample was run in triplicate for each target. For the initial run, three different template concentrations for each sample were analyzed to determine which gave a Cp that best fit within the standard curve. In the subsequent run, this concentration was analyzed in duplicate and the mean of all three Cp values were used in the analysis. Data was analyzed using the LightCycler Data Analysis Software version 3.5.5 and the "2nd derivative" algorithm.

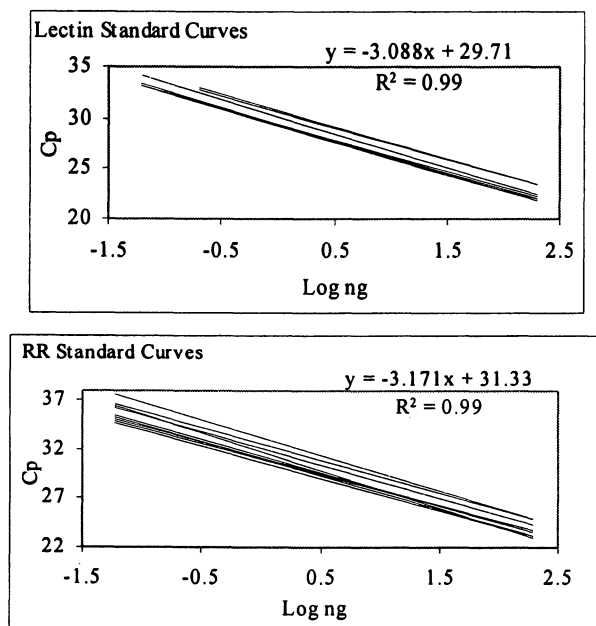


Figure 1. Standard curves for both lectin and RR PCRs each performed 9 times. Calculated E values from each set of data are 2.07 (RR) and 2.11 (Lectin)

Table III Mean Cp and SD Values for Lectin and RR Standards

DNA (ng)	Lectin Standard PCR		RR Standard PCR	
	Mean Cp	SD	Mean Cp	SD
200	22.53	0.74	23.92	0.67
100	23.61	0.77	25.06	0.62
25	25.35	0.65	26.79	0.71
6.25	27.17	0.58	28.82	0.83
0.78	30.27	0.72	31.92	0.88
0.06	33.36	0.32	35.02	0.39

Determination of percent RR soy relative to total soy DNA in food samples

Two standard curves were generated from 7 replicates of the lectin and RR assays. The equations from the linear regressions of these curves were used to calculate the ng of lectin or RR DNA in the food samples based on the mean of the crossing points. The percent RR soy was determined from the ratio of ng of RR divided by the ng of lectin multiplied by 100. Some foods required more template in the RR reaction compared to the lectin reaction in order to bring the crossing points within the range of the standard curve (4 x template: meat alternatives 1 and 2, soup mix; 8 x template: protein bar 1 and 2; 20 x template: tofu and 5% Fluka CRM) and therefore the RR values had to be divided by these factors before the ratio to lectin could be made.

Results

Classification of Soy Containing Foods

The 39 foods chosen for this study had soy listed as an ingredient(s) on the label and represent of a wide range of soy containing foods. The soy ingredient(s) included whole bean, flour, de-fatted flour, powder, soy protein, soy milk, and lecithin. Table 1 lists the soy foods and the degree of processing of the soy ingredient(s). Degree of processing was based on previously published descriptions of soybean products and their methods of preparation (9,21,22). Foods with a number of different forms of soy in the ingredients were classified based on the least processed form.

DNA Extraction

All samples were extracted using the modified Wizard™ method. Samples that gave no or poor lectin amplification by qualitative PCR were re-extracted with the Qiagen Stool Kit (soy sauce, chocolate pudding, miso, gravy mix 1, natto, cracker 1, and cheesies). To determine the degree of degradation, and therefore the quality of the extracted DNA, approximately 200 ng of each DNA sample was visualized by agarose gel electrophoresis (Figure 2).

Many of the food extracts contained degraded DNA with an average fragment size of less than 564 bp. Yields from the various foods ranged from 0.15 µg/100 mg (soy sauce) to 38 µg/100 mg textured vegetable protein (TVP). The cheesies and miso samples extracted with the Qiagen Stool Kit both had an absorbance at 260 nm less than 0.1 and therefore could not be measured accurately. All the samples had $A_{260/280}$ ratios between 1.7 - 1.9 with the exception of soy sauce (2.3), cheesies (2.0), and coffee whitener (2.0).

Qualitative PCR

To determine if there was amplifiable soy DNA in the food extracts, PCR was performed using the lectin specific primers GMO3 and GMO4 (9). The LOD for the lectin assay was 50 pg (44 genome copies) of total soy DNA (Figure 3).

Figure 4A shows the amplified lectin band (118 bp) for each of the soy foods. Lectin PCR products from miso, gravy mix 1, cheesies, and natto were weak and there were no clear amplification products of the expected size from the cracker 1 sample. These foods were re-extracted using the Qiagen Stool Kit which improved amplification from cheesies, miso, and cracker 1 (Figure 4A). The 39 food samples were subsequently analyzed using primers RR04 and RRO5 (9) specific for the CTP4-CP4EPSPS junction of the RR transgene. Of these 39 samples, 28 tested positive for RR. (Figure 4B). The LOD for this assay was 10 pg (8 genome copies) of RR soy DNA (data not shown). Three samples: gravy mix 1, miso and cheesies, which amplified poorly with the lectin specific primers, weakly amplified with the RR specific primers. RR could not be detected in natto and cracker 1 which were also only weakly amplified with the lectin primers. The dried (organic) soybean, soybean pâté, soup, meal replacement beverage, soy nut snack, nutritional supplement bar, crispbread, chocolate pudding, and soy sauce which strongly amplified with the lectin primers also tested negative in the RR PCR. Bands with a higher molecular weight than expected were observed in miso, cracker 1, coffee whitener, and crispbread, which likely is due non-specific amplification from rye or wheat in these samples (unpublished results).

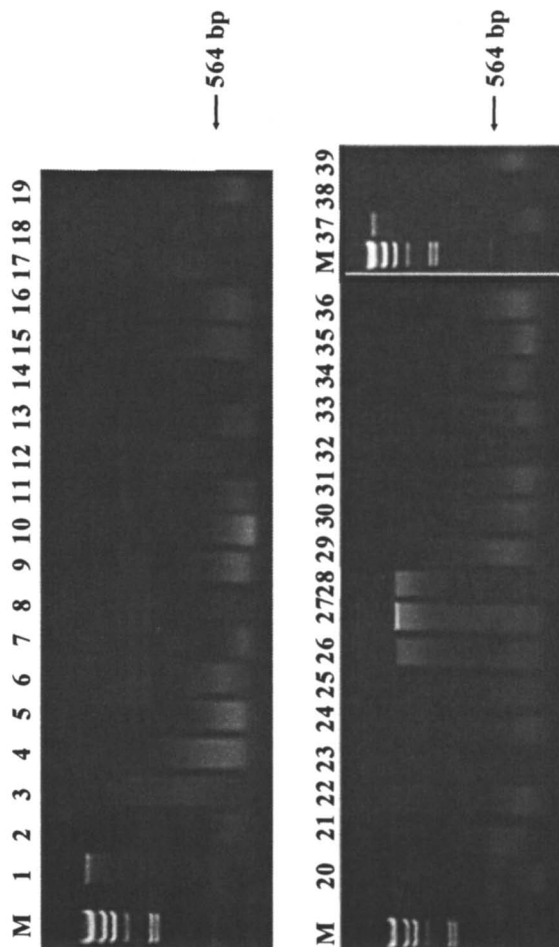


Figure 2. Agarose gel electrophoresis of nucleic acids extracted from the soy foods using the modified Wizard method unless otherwise stated. Lane 1-dried (organic) soybean, 2-soy nut spread, 3-tofu, 4-bread 2, 5-bread 1, 6-meat alternative 1, 7-soybean pâté, 8-cracker 2, 9-meat alternative 2, 10-soup, 11-meat alternative 3, 12-yogurt, 13-infant formula 1, 14-meal replacement beverage, 15-protein bar 2, 16-protein bar 1, 17-coffee whitener, 18-soy beverage, 19-infant formula 2, 20-miso (Qiagen Stool Kit), 21-TVP, 22-gravy mix 2, 23-frozen dessert, 24-soy nut snack, 25-nutritional supplement bar, 26-soup mix, 27-gravy mix 1, 28-simulated bacon bits, 29-crispbread, 30-cracker 1 (Qiagen Stool Kit), 31-cheese, 32-cheesies (Qiagen Stool Kit), 33-roasted soy nuts, 34-natto, 35-biscuit, 36-flour, 37-SI4M7 soybean, 38-soy sauce (Qiagen Stool Kit), 39-chocolate pudding (Qiagen Stool Kit). M: molecular weight marker II (Roche). (reproduced with permission from reference 30.

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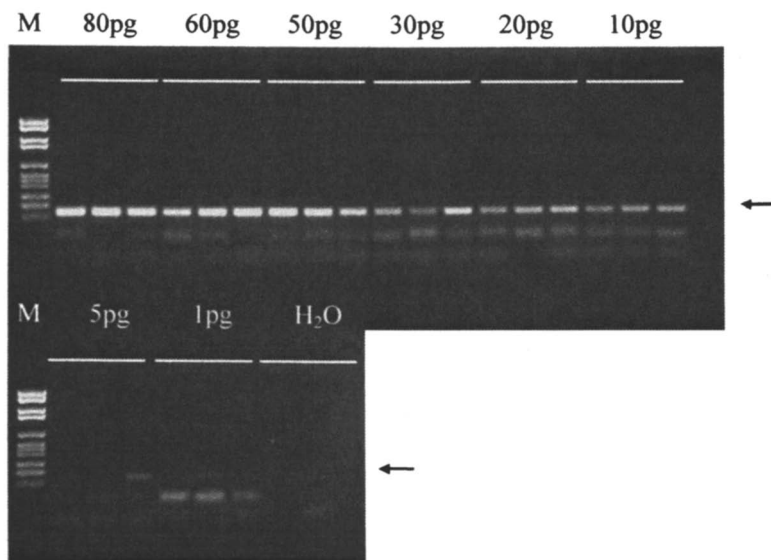


Figure 3. Limit of detect (LOD) for RR PCR. Determined by assaying dilutions of genomic soyabean DNA in triplicate. 10 pg genomic DNA was the lowest dilution that gave a positive signal in all replicates (arrow) and was defined as the LOD. Based on a 1C value of 1.13pg for canola, the LOD is calculated to be 8 haploid copies.

Quantitative PCR

The 28 food samples which tested positive for the RR trait were further analyzed by quantitative real-time PCR. The standard curves for both the lectin and the RR assays were made from serially diluted S14M7 DNA. Dilutions in the range of 200 ng - 0.06 ng were used to generate the standard curves. Nine inter-assays for lectin and RR were performed, and the mean efficiencies (E) calculated to be 2.11 and 2.07, respectively (Figure 1, Table III). Data was linear through the range of dilutions, therefore 0.06 ng (53 genome copies) was defined as the limit of quantification (LOQ). This limit is well above the experimentally determined LOQ of 30 copies reported by Berdal and Holst-Jensen (2,9). For a pure (100%) soy product, the LOQ expressed as %RR/soy can be defined as the lowest quantifiable amount of RR soy DNA (0.06 ng) divided by the highest quantifiable amount of soy DNA (200 ng) multiplied by 100 (eg., $0.06 \text{ ng}/200 \text{ ng} \times 100 = 0.03\%$). The limits of detection for the lectin and RR quantitative assays were not determined. The total soy DNA in the foods was determined using

primers LecLC-F, LecLC-R and probes LecLCFITC-1, LecLCR705-1 and the total RR DNA in the food products was determined using primers RRLC-F20, RRLC-R15 and probes RRSoyLCFITC-1, RRSoyLC640-1 (Table II). Initially, three serial dilutions of the DNA food extracts (25 ng -200 ng) were analyzed to determine E for both the lectin and RR assays. A subsequent assay in duplicate for each food extract was used to calculate the % RR DNA relative to total soy DNA. Table IV summarizes the results for the foods that were above the LOQ for the RR quantitative PCR assay. The ng of target DNA was determined by comparing the mean crossing point of each sample to the standard curve. Cheesies, miso, and gravy mix 1 had a weak lectin amplicon in the qualitative PCR (Figure 4A) and were below the limit of quantification for the lectin in the real-time PCR (data not shown). For this reason, the amount of RR soy in these samples was not quantified. Fourteen of the 36 food samples contained quantifiable levels of RR soy that ranged from 0.03% to 87%. Foods that contained levels of RR soy greater than 5% included TVP, frozen dessert, infant formula 1, simulated bacon bits, bread 1, bread 2, biscuit, and gravy mix 2. Foods that contained less than 5% RR soy included meat alternative 1, meat alternative 3, tofu, protein bar 1, protein bar 2, and soup mix. In order for the quantitative results for these foods to fall within the RR standard curve, 4 - 20 times more DNA had to be added to the RR reactions compared to the lectin reactions. Eleven samples contained only trace amounts of amplifiable RR specific DNA which could not be quantified in the real-time PCR assays. These included yogurt, boiled soybean, flour, cheese, infant formula 2, soy beverage, roasted soy nuts, cracker 2, coffee whitener, meat alternative 2, and soy nut spread. A positive control containing 5% RR Soy (Fluka) was analyzed together with the samples and calculated to contain 4.6% RR/total soy DNA. GeMMA sample GeM18, meat pâté was used as a processed food control. GEM18 was given an assigned value of 8.5% RR soy by GeMMA and was calculated in this study to contain 6.7% RR soy.

Calculations of percent RR soy in the food samples were based on the assumption that the real-time PCR amplification E values of the standards and sample were the same. To determine the validity of this assumption, E values of the food samples were calculated and compared to the standards. Whereas E values for the standards are based on a standard curve consisting of 6 dilutions spanning a little more than 3 orders of magnitude (200ng to 0.06ng) repeated 9 times for a total of 54 data points, each food sample consisted of only 3 dilutions spanning less than 1 order of magnitude repeated 3 times for a total of 9 data points. The amplification efficiencies of the food samples ranged from $1.81 \leq E \leq 2.11$ for lectin, and $1.74 \leq E \leq 2.31$ for RR (Table V).

For the lectin reaction, all samples had E values equal or less than the standard. For the RR assay, meat alternative 1, protein bar 1, and protein bar 2 had E values greater than the standard ($E = 2.31, 2.18$ and 2.13) with the other food samples having E values equal or less than the standard (Table IV). The

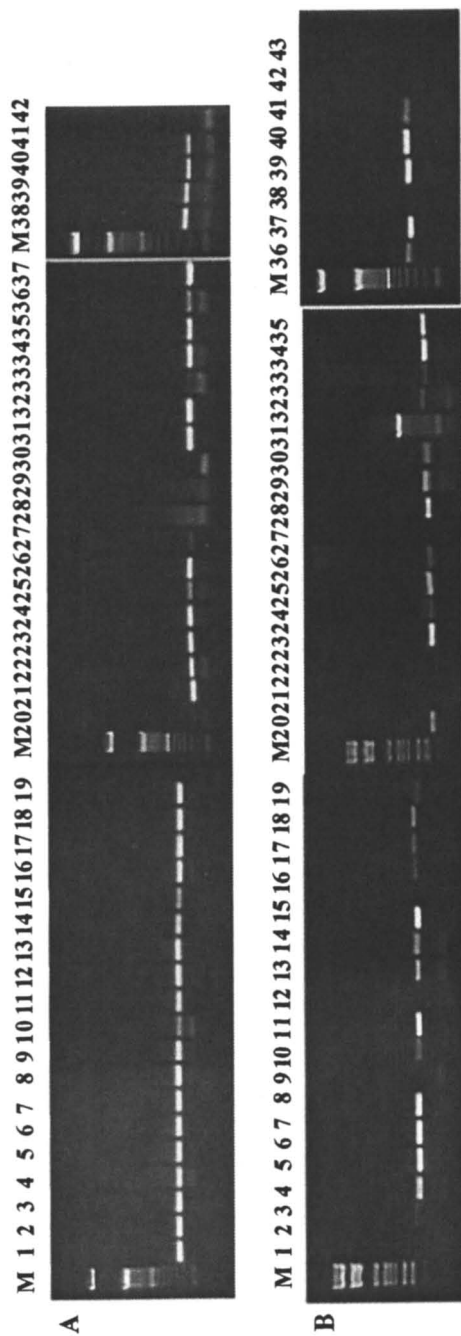


Figure 4. (A) Qualitative PCR analysis of lectin and Roundup Ready® DNA in food samples. The 118 bp PCR product represents the amplified region of the lectin gene using primers GMO3 and GMO4. Lane 1-dried (organic) soybean, 2-soy nut spread, 3-tofu, 4-bread 1, 6-meat alternative 1, 7-soybean pâté, 8-cracker 2, 9-meat alternative 2, 10-soup, 11-meat alternative 3, 12-yogurt, 13-infant formula 1, 14-meal replacement beverage, 15-protein bar 2, 16-protein bar 1, 17-coffee whitener, 18-soy beverage, 19-infant formula 2, 20-miso, 21-TVP, 22-gravy mix 2, 23-frozen dessert, 24-soy nut snack, 25-nutritional supplement bar, 26-soup mix, 27-gravy mix 1, 28-cracker 1, 29-boiled soybean, 30-simulated bacon bits, 31-natto, 32-biscuit, 33-chocolate pudding, 34-soy sauce, 35-positive control (SI14M7), 36-flour, 37-cheesies, 38-cheese, 39-crispbread, 40-roasted soy nuts, 41-positive control (SI14M7), 42-water control (no DNA). M: 50 bp DNA ladder (Invitrogen). (B) The 180 bp PCR product represents the CTP4 - CP4EPSPS junction of the Roundup Ready® transgene using primers RR04 and RR05. Lane 1-dried (organic) soybean, 2-soy nut spread, 3-tofu, 4-bread 2, 5-bread 1, 6-meat alternative 1, 7-soybean pâté, 8-cracker 2, 9-positive control (SI14M7), 10-soup, 11-meat alternative 3, 12-yogurt, 13-infant formula 1, 14-meal replacement beverage, 15-protein bar 2, 16-protein bar 1, 17-infant formula 2, 18-cheesies, 19-gravy mix 2, 20-soy nut snack, 21-nutritional supplement bar, 22-soup mix, 23-miso, 24-cracker 1, 25-TVP, 26-meat alternative 2, 27-water (no DNA), 28-coffee whitener, 29-soy beverage, 30-cheese, 31-crispbread, 32-roasted soy nuts, 33-gravy mix 1, 34-frozen dessert, M: molecular weight marker VI (Roche) 35-boiled soybeans, 36-simulated bacon bits, 37-natto, 38-biscuits, 39-positive control (SI14M7), 40-flour, 41-chocolate pudding, 42-soy sauce, 43-water (no DNA). M: 50 bp ladder (Invitrogen). (Reproduced with permission from reference 30. Copyright 2004 American Chemical Society.)

Table IV. Quantitative Results for Soy Containing Foods Positive for RR

Food	Target	Cp	SD	Target (ng)	%RR/Soy	Corr. %RR/Soy
TVP	Lectin	25.58	0.10	21.7		
	RR	27.83	0.11	12.7	58	
Meat alternative 1	Lectin	24.77	0.06	39.8		
	RR ^c	33.14	0.08	0.067	0.17	<LOQ
Soup mix	Lectin	25.62	0.03	21.1		
	RR ^c	30.37	0.18	0.502	2.4	
Meat alternative 3	Lectin	26.53	0.08	10.7		
	RR ^c	33.80	0.29	0.042	0.39	5.0
Protein bar 1	Lectin	24.65	0.06	43.5		
	RR ^d	33.78	0.44	0.021	0.05	<LOQ
Protein bar 2	Lectin	25.16	0.11	29.7		
	RR ^d	34.61	0.33	0.012	0.04	<LOQ
Tofu	Lectin	25.39	0.09	25.1		
	RR ^e	33.87	0.38	0.008	0.03	0.43
Sim. bacon bits	Lectin	23.55	0.16	98.8		
	RR	25.85	0.03	53.5	54	
Frozen dessert	Lectin	26.44	0.20	11.5		
	RR	28.73	0.05	6.60	58	
Infant formula 1	Lectin	25.58	0.05	21.7		
	RR	27.73	0.13	13.7	63	
Bread 1	Lectin	28.30	0.37	2.86		
	RR	30.27	0.21	2.16	75	
Bread 2	Lectin	28.83	0.12	1.93		
	RR	30.81	0.18	1.46	76	2477
Biscuit	Lectin	26.41	0.10	11.7		
	RR	29.30	0.05	4.37	37	
Gravy mix 2	Lectin	27.60	0.11	4.82		
	RR	29.36	0.12	4.18	87	
5% Fluka	Lectin	25.30	0.05	26.8		
	RR ^e	26.92	0.06	1.23	4.6	
GeMMA Meat Pâté	Lectin	28.54	0.07	2.39		
	RR	33.85	0.18	0.16	6.7	

^aCp:crossing point (mean of 9 replicates for standard curves and mean of three replicates for the food samples), ^bSD:standard deviation, ^cFour times more template used in the RR reaction compared to the lectin reaction, ^dEight times more template used in the RR reaction compared to the lectin reaction, ^eTwenty times more template used in the RR reaction compared to the lectin reaction. (Reproduced with permission from reference 30. Copyright 2004 American Chemical Society.)

Table V. Lectin and RR PCR Efficiencies for Soy Containing Foods Positive for RR

Food	Lectin E ^a	ΔE^b	RR E ^c	ΔE^d	$\Delta\Delta E^{b-d}$
TVP	1.95	0.16	1.85	0.22	0.06
Meat alternative 1	2.11	0.00	2.31	-0.24	0.24
Soup mix	1.93	0.18	1.81	0.26	0.08
Meat alternative 3	2.05	0.06	1.88	0.19	0.13
Protein bar 1	1.95	0.16	2.18	-0.11	0.27
Protein bar 2	1.90	0.21	2.13	-0.06	0.27
Tofu	1.98	0.13	1.83	0.24	0.11
Simulated bacon bits	2.08	0.03	1.94	0.13	0.10
Frozen dessert	1.81	0.30	1.77	0.30	0.00
Infant formula 1	2.01	0.10	1.96	0.11	0.01
Bread 1	2.01	0.10	2.05	0.02	0.08
Bread 2	1.98	0.13	1.74	0.33	0.20
Biscuit	1.97	0.14	1.97	0.10	0.04
Gravy mix 2	1.95	0.16	1.86	0.21	0.05
5% Fluka	1.97	0.14	1.95	0.12	0.02

^aE: efficiency of the lectin PCR calculated from the slope of logarithm of the ng of template versus Cp for three dilutions of the sample ($E = 10^{(-1/\text{slope}^e)}$), ^b ΔE : difference in efficiency between the food sample and the lectin standard curve (2.11, calculated from the slope in Table 3), ^cE: efficiency of the RR PCR for three dilutions of the sample, ^d ΔE : difference in efficiency between the food sample and the RR standard curve (2.07, calculated from the slope in Table 3), ^{b-d} $\Delta\Delta E$: the difference in efficiency of the food samples between the lectin and RR PCR.

difference in E between food sample and standard for each of the lectin and RR assays is expressed as ΔE . For most of the foods, ΔE values for the RR assay deviated from the standard to a greater degree than the lectin assay. The difference in ΔE values between the lectin and RR assays for each food sample is expressed as $\Delta\Delta E$. A low value of $\Delta\Delta E$, indicates that the efficiencies for the lectin and RR assays for a sample deviated from the standard to the same degree. Eight food samples and the 5% Fluka standard had $\Delta\Delta E$ values of 0.1 or less. The remaining 6 foods had $\Delta\Delta E$ values greater than 0.1 (Table V). For foods samples with $\Delta\Delta E$ values greater than 0.1, a correction factor:

$$C_{p_{corr}} = C_{p_{meas}} \cdot \frac{\log(E_f)}{\log(E_s)} \quad \text{Equation (1) (23)}$$

where E_f is the efficiency measured for the food sample and E_s is the efficiency for the corresponding standard, was used to calculate corrected percent RR values (Table V). When this correction factor was applied, meat alternative 1, and protein bars 1 and 2, were no longer within the LOQ, for bread 2 the percent RR values rose from 76% to over 2400%, and for 1 sample (meat alternative 3) the percent RR soy changed from 0.39% to 5.0%, which could alter the classification of this food with respect to a proposed Canadian labeling threshold of 5%.

Discussion

With the introduction of legislations concerning the labeling of foods containing GM products there is a requirement to develop methods to detect and quantify the presence of these products. It is rather unfortunate that legislation was initially introduced in the absence of sound scientific input on the technical feasibility of meeting the set legislative regulations. The majority of studies to date have been limited to detection/quantification of GM products in idealized control samples which may or may not be representative of real-world food samples. In this study, conventional and real-time PCR methods were used to analyze the GM content, specifically RR soy, in soy containing foods obtained from local markets. There are several advantages to a nucleic acid analysis method compared to methods that detect the novel proteins produced by the GM plant. For example, nucleic acids are generally more stable than proteins to the degradating effects commonly used in food processing. Therefore, there is a greater likelihood of detecting GM products in processed foods by PCR compared to protein based methods such as enzyme-linked-immunosorbant-assay (ELISA). Also, different GM plant varieties can produce varying levels of

the transgenic protein, which makes quantification virtually impossible. Quantitative PCR, and in particular real-time quantitative PCR, is still lacking as a rigorous analytical tool, particularly at the level required by GM regulations under even the best case scenario. Quantitative PCR analysis of food samples is far from a best case scenario with significant issues that can effect the reliability of the method. In particular, preparation of the sample (DNA extraction) and the sample matrix (including the degree of processing of the sample) can have a huge effect on the quality and quantity of DNA that can be isolated from the sample for analysis. Other issues include the choice/availability of reference material, method validation, and biological/instrumental errors (18). As a result, the technical difficulties towards obtaining an accurate assessment of the GM content present in foods by PCR is a challenge for even the most highly experienced technical expertise, that in some cases may not be attainable.

Food Processing Effects

In general, it can be assumed that increasing levels of processing results in a decrease in the amount and quality of DNA. This can have a significant effect on the ability to detect/quantify the amount of DNA by PCR. Agarose gel electrophoresis of the DNA extracted from the food samples showed that most contained highly degraded DNA as expected. In order to amplify effectively from this DNA it is important that the amplified fragments be short. In the assays used here, PCR fragments ranged from 114-180 bp in length. For 2 of the most highly processed food products tested, miso and cheesies, both lectin and RR could be detected but not quantified. This is likely due to the very low yields of DNA. In order to obtain sufficient DNA for quantitative analysis, larger amounts of the food sample would have to be used in the DNA extraction procedure. For both miso and cheesies this would likely have resulted in relatively high percent levels of RR since both could be detected in the small amount of DNA extracted.

Extraction Efficiencies and DNA Purification

Previous studies have reported difficulty extracting amplifiable DNA from soy sauce (4,9,15). However, using the Qiagen Stool Kit, enough DNA was obtained for analysis. DNA from food products containing chocolate have also been problematic (29,9,14,15,16). While DNA obtained from chocolate pudding using the Wizard extracted method could not be amplified, DNA obtained using the Qiagen Stool Kit could. DNA extracted using the modified Wizard method from protein bar 1 and 2, which list cocoa as an ingredient, could be amplified in

the PCR assays. The proportion of cocoa in the protein bars may be low enough that the PCR-inhibitory compounds are not enough to significantly affect the PCR. The Qiagen Stool Kit also improved the quantitative PCR from cheesies, miso and cracker, although the amount of DNA obtained was too low for accurate measurement from cheesies and miso. From these examples, it can be seen that a single extraction method is not necessarily appropriate for all food types, and that some experimentation may be required using different extraction methods, or modifications of these methods to maximize either the DNA quality, quantity, or both from any particular food matrix.

Lectin could be weakly detected in gravy mix 1, natto and cracker 1 and RR could only be detected in gravy mix 1. Soy in the form of oil/hsp in gravy mix 1, likely contains only low amounts of degraded soy DNA which does not correspond to the high molecular weight DNA extracted from gravy mix 1 (Figure 2). The majority of DNA from these these foods probably originates from ingredients other than soy. Infant formula 1 and 2 contained the same type of soy ingredients, soy milk, isp and oil and both showed comparable DNA fragment sizes on the agarose gel (Figure 2). Infant formula 1 contained 63% RR soy while infant formula 2, which contained about half as much total soy as infant formula 1, was below the LOQ for the RR assay. It would therefore appear that very little of the soy in infant formula 2 was RR soy, whereas in infant formula 1 a high percentage of the soy was derived from RR soy. There is a large difference in the quality of DNA in the extracts among the foods that were shown to contain higher levels of RR soy. For example, the DNA extracted from the frozen dessert was so degraded that it was not visible on the agarose gel whereas breads 1 and 2 have a greater abundance of higher molecular weight DNA fragments (Figure 2).

These results highlight problems of accurately measuring the amount of soy that is highly processed and/or present in a complex food product. In the first situation, highly processed soy products require a very large sample in order to obtain sufficient DNA for analysis. In the second situation where soy is an ingredient of a complex food product, the total amount of DNA extracted may not be limiting, but the quality and quantity of the DNA to be amplified is limiting. If the food contains soy as a highly processed ingredient together with other food ingredients, these 2 factors are compounded. This has significant implications for compliance labeling purposes. It has been suggested that certain processed food ingredients that contain little or no amplifiable DNA be exempt from testing/labeling (24). However, this raises additional questions on how products to be exempt are to be defined. The issues of sample matrix and DNA extraction are complex, with each food representing a potentially unique situation and much work still needs to be undertaken. However, a relatively simple (technically) solution would be to test for genetically modified soy before processing or before it is added as a food ingredient. This would significantly reduce the types of sample matrices that would need to be analyzed and increase the possibility of developing appropriate control samples (see below). This

would however, require significant changes in the documentation, and verification of food products entering the market, some of which are already being considered or implemented by certain countries.

Assay Controls

To test the accuracy of quantitative tests, two known controls were analyzed. The 5% Roundup Ready[®] soybean powder (Fluka), IRMM certified reference standard was calculated to contain 4.6% RR soy, an error of only 8%. The second control, GeM18 meat pâté sample, is more representative of the processed foods analyzed in this study. To summarize, the composition and preparation of this sample was as follows. A 6-7% Roundup Ready[®] soy w/w of total soy flour mix was added to other pâté ingredients (pig liver, pork belly, water, skimmed milk powder, salt, sorbic acid, sodium polyphosphate, pepper, onion powder, and monosodium glutamate) at a level of 5%, mixed and baked at 130°C for 1 hour and then at 150°C until a core temp of 80°C was obtained. Homogeneity testing of the pâté samples by GeMMA (10 sub-samples, 2 replicates of each) gave a mean RR soy/total soy value of 5.5% with a range from 3.8 to 8.9%. The assigned percent RR value for the pâté, based on the consensus mean of 81 laboratories participating in GeM18 proficiency panel was 8.5%, with acceptable individual results (based on z-scores) ranging from 3.4% to 21.4%. In this study, the meat pâté sample was calculated to contain 6.7% RR soy/total soy which is close to both the mean value determined by homogeneity testing and the assigned value. Close agreement of the experimentally determined and accepted values for both the RR flour and processed food controls gives confidence that the values obtained for the unknown processed food samples are accurate.

Amplification Efficiencies

For precise quantification by real-time PCR, amplification efficiencies of the standards and sample must be the same (2,9). For many quantitative PCR experiments this assumption is made but not tested. Where the source material for the standards and the samples are of the same matrix (e.g., both source and sample DNA are extracted from whole soybean) this assumption is close enough to be valid, assuming a high level of technical competency in the performance of the assay. Calculations show that differences in PCR efficiencies of only 5% can result in a 2 fold difference in measurement after 26 cycles (25). In this study, the standards consisted of high molecular weight DNA extracted from unprocessed soybean. The only current certified reference standards available on the market for quantitative PCR analysis of RR soy are ground flour mixtures produced by the Joint Research Centre - IRMM. In contrast, many of the food

samples analyzed in this study contained degraded soy DNA as a result of processing. In addition, soy was often only one of several food ingredients in a complex food sample. Essentially, each food represents a different matrix, often very different from the unprocessed soybean standard, used in this study. Each food potentially contains compounds that are not removed during the DNA extraction procedure which could affect the efficiency of the PCR. There are also potential problems when using a different extraction method for a particular food and the reference standard used. Ideally, the same methods should be used for both. Therefore, for the quantitative PCR analysis of GM foods, the assumption cannot be made that efficiencies of standards and samples are the same.

Several methods have been reported to compare differences in PCR efficiencies (E) between standard and sample, each with its own advantages and disadvantages. A series of multiplex reactions can be used to calculate E (26); E values can be determined based on the kinetics of the individual sample reactions (27), or calculated based on a series of dilutions (23). In this study, E values were based on a series of dilutions. The mean calculated values for the standards were $E=2.11$ (lectin) and $E=2.07$ (RR). Each value is based on a total of 54 data points spanning a dilution range of 3×10^3 , which is statistically twice the number required for an accurate determination of E over this range. E values for the food samples, however, are based on only 9 data points for over a dilution range of less than 1 order of magnitude. As the dilution range decreases, the number of data points that are required for an accurate determination of E increases. Whereas a dilution range of 10^3 requires only 27 data points, a dilution range of 10^1 would require 240 data points. Clearly, this is not practical! It is also not possible to significantly expand the dilution range by further diluting the sample due to the limited amount of soy DNA extracted from the foods, or by increasing the total amount of DNA used in the assay due to inhibitory effects. As a result, calculated E values for the foods are an approximation only, and most likely over estimate the difference in E values between the standards and foods. E values for the foods differed from the standards (ΔE) by as much as 0.30 (lectin) and 0.33 (RR). In most cases, E values for the food samples were less than that of the standard. This suggests that inhibitors were present in the food DNA extractions. If the reduction in E of the sample was due to the presence of inhibitors it might be expected that both the lectin and RR assays would be equally effected. This can be determined from the difference in the ΔE values of the lectin and RR assays ($\Delta\Delta E$). A small $\Delta\Delta E$ for a food sample indicates that the E values for both the lectin and RR assays differed from the E values of the standard by the same amount. Eight foods and the 5% Fluka standard had $\Delta\Delta E$ values of ≤ 0.1 . For example, in the frozen dessert sample, Cp values for lectin and RR are 26.44 and 28.73 and ΔE values were 0.30 and 0.30, respectively for the frozen dessert. For both reactions this would result in a calculated underestimation of the amount of lectin and RR by

approximately 80 X. However, the $\Delta\Delta E$ value is 0.00. Therefore, because the efficiencies of the lectin and RR assays deviate from the mean value of the standards by the same degree, the ratio of the measured amounts of each (percent RR) is still valid, even if the absolute values are incorrect. Some of the foods had relatively high $\Delta\Delta E$ values (≥ 0.10) which could indicate that the %RR determined is incorrect. High $\Delta\Delta E$ could be due to a number of factors, for example differences in the robustness of the lectin and RR assays. A greater variation in the RR assay for the standards and the food samples was observed compared to the lectin assay which could indicate that the RR assay is less robust. However, greater variations in the food quantitative RR assay may also be because the amount of RR relative to the total amount of soy can be much lower. Meat alternative 1, protein bar 1, and protein bar 2 contained low measured amounts of RR (0.04-0.39%) and had the largest $\Delta\Delta E$ values (0.24 - 0.27). To try and compensate for foods with $\Delta\Delta E$ values ≥ 0.10 , a correction factor was applied (equation 1). When this correction factor was applied to meat alternative 1, and protein bars 1 and 2, the amount of RR present was calculated to be below the LOQ. It has been demonstrated that low concentrations of target can lead to a calculation artifact with the LightCycler (28). The curve of C_p vs Log of concentration, at low target concentrations, is no longer linear but parabolic, and calculations of E within this range would give artificially high values if a correction factor wasn't used. This appears to be the case for meat alternative 1, and protein bars 1 and 2.

Applying the correction factor to bread 2 ($\Delta\Delta E = 0.20$) resulted in an increase in %RR from 76% to 2477% which clearly is not possible. This result is probably due to an error in the calculation of E for bread 2 due to the insufficient number of data points. Bread 2 contained the least amount of soy of all foods quantified (less than 1% of the total DNA extracted could be attributed to soy), slight errors in measurement at these concentrations would have a large effect on calculated E values. The correction factor was also applied to meat alternative 3 ($\Delta\Delta E = 0.13$) and tofu ($\Delta\Delta E = 0.11$) resulting in a measured increase in %RR of about 10X from 0.39 % to 5.0 % and 0.03% to 0.43%, respectively. For all corrected samples, the change in calculated %RR would not have affected the classification and subsequent labeling under the newly proposed Canadian voluntary labeling guide for genetically modified products. The usefulness of the correction factor is directly related to the accuracy with which E for the foods can be determined. Unfortunately, there is not a fully satisfactory and practical method for determining E at this time. The method used here gives only an approximation that probably over estimates the change in E relative to the standards. As such it presently could be useful in determining the amount of error in the final % values until more accurate methods of determining E for the foods have been developed.

Summary

For the majority of foods tested, unequivocal results could be obtained for the presence/absence of soy and RR soy. These results are encouraging and suggest that even for foods with a relatively high amount of processing, detection of GM products is possible. In the quantitative assays, 2 main groups were observed, foods with trace amounts of RR soy $\leq 0.4\%$, suggestive of adventitious contamination, and foods with relatively high levels of RR soy $\geq 37\%$, indicating that RR soy was an intended ingredient. For the purposes of this study and labeling regulations, this made categorizing the foods relatively straightforward if the labeling threshold was set at 5% (e.g. Canada) as minor errors in the analysis would not have a significant effect on how the product would be labeled. However, many questions remain to be answered before reliable routine testing of foods can take place. For example, what is the accuracy and how much error is present/acceptable in the calculated %RR values? Following this, at what point is a food considered positive for the presence of GM product and should be labeled? Considering the potentially large amounts of error present in the PCR method for the analytical quantification of GM events and the uncertainty involved, it is likely that the amount of error in the measurement is so great as to make the threshold regulatory values set by some countries almost meaningless. For example, the EU stipulates GM presence of 0.9% or more must be labeled. For all practical purposes, it would be very difficult to accurately determine whether anything testing positive is below this level and should not be labeled. Therefore, to be conservative, a positive test for GM should be considered a positive test for labeling. Conversely, if an error value can be reasonably determined, for example $\pm 2\%$, a product would have to test at least $0.9 + 2.0 = 2.9\%$ to be considered a positive. To help solve these issues, methods need to be developed for a more accurate determination of E for the foods being tested so that a correction factor can be more confidently applied. Failing that, an alternative would be to develop reference standards that are more representative of the foods being tested.

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Chapter 3

Enantiomeric Analysis as a Tool for Authentication of Foods and Beverages

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Numerous chiral compounds occur in foods and beverages. The fact that enantiomeric composition can be used to distinguish biological processes from chemical processes has been recognized for over a century. However, analysis of enantiomeric purity or enantiomeric ratios of specific compounds remained challenging, requiring large samples, derivatization of functional groups, or separations based on physical process such as crystallization. Development of cyclodextrin-based GC stationary phases in the 1970-80's provided the ability to directly separate a large number of underivatized chiral compounds present at low concentrations. Now, chiral analysis is frequently used for authentication of foods and beverages with respect to source ("natural"/synthetic), geographic origin, processing/aging treatments, and formation mechanisms (chemical/ enzymatic). This paper will use selected examples to demonstrate the application of enantiomeric analysis for food and beverage authentication.

Wine has traditionally been labeled and marketed according to geographic origin, grape variety, and vintage year (or age). However, in the United States other foods and beverages have only recently begun to exploit these attributes for marketing, economic, or food safety reasons. Voluntary and/or mandatory labeling for point-of origin is now being explored for many foods (1) and has highlighted the need for improved analytical tools for confirming the authenticity of these claims.

Enantioselective analysis has been used to assess technological processes, contamination, adulteration, and aging of foods and beverages (reviewed by 2). Because biological processes often use enzymes for synthesis of many compounds, the products of these reactions are often produced in specific enantiomeric ratios. The predominance of L-amino acids and D-sugars in many biological systems are examples of biological processes which produce optically pure compounds. Chemical synthetic routes, on the other hand, often yield racemic product mixtures and so "natural" vs. "synthetic" sources for many compounds can be readily distinguished.

Although the existence of chiral compounds was recognized by Pasteur in the mid 1800's, the use of chiral measurement as an analytical tool was limited until the mid 1970's and early 1980's with the development of cyclodextrin-based stationary phases for gas chromatographic separations. These stationary phases allowed for the direct analysis of volatile chiral compounds without the need for highly purified chiral derivatizing agents. Modifications to the hydroxyl groups on the glucose molecules of the cyclodextrins (eg., derivatization with methyl, pentyl, acetyl, trifluoroacetyl, and *t*-butyldimethylsilyl groups) and addition of the cyclodextrins to conventional stationary phases have allowed for improved chromatographic properties, analysis at low operating temperatures, and a range of separation mechanisms based on both the size of the cyclodextrin cavity and surface interactions (3-6). The cyclodextrins are now widely used, either as chromatographic stationary phases or as mobile phase additives with HPLC and capillary electrophoresis, for enantiomeric analysis of food and beverage composition.

This paper will discuss the applications and limitations of enantiomeric analysis as a tool for authentication of foods, beverages, and essential oils. Using selected examples, we will discuss past and current research on food authentication with respect to 1) identification of source, 2) varietal characterization, 3) confirmation of geographic origin, and 4) confirmation of processing or product age.

Identification of Source: Natural vs Synthetic

One of the most common applications of enantiomeric analysis is as a tool to confirm whether the source of a food or flavoring agent is from a biological or synthetic source. A "Naturalness Decision Tree" has been proposed by

Mussinan and Hoffman (7) in which enantiomeric analysis, in combination with isotope ratio mass spectrometry (IRMS) and NMR, can be effectively used to confirm product source (IRMS and NMR are discussed in other chapters of this proceedings). A typical example is the predominance of R-(+)- α -ionone in natural raspberry flavors compared with the presence of a racemic mixture of R-(+)/S-(-)- α -ionone in synthetic flavorings (8). Ebeler et al. (9), using a rapid solid phase microextraction (SPME) sampling technique, showed that α -ionone isomers in flavored alcoholic beverages could be readily screened using GC with cyclodextrin stationary phases, and products with “natural” and “artificial” flavoring could be confirmed based on enantiomeric composition. Similarly, enantiomeric composition of terpenes in citrus flavored carbonated beverages and essential oils has also been used to authenticate the flavoring source (10-12). As shown by Mosandl et al. (13, 14), the S-enantiomers of 2-methylbutanoic acid and its methyl ester predominate in apples and apple-containing foods, therefore the presence of 2-methylbutanoic acid (and ester) racemates may be an indicator of synthetic flavoring. Many other applications for authenticity control of flavor compounds and essential oils have been studied and are reviewed by Mosandl (5) and Marchelli et al. (2).

While seemingly straightforward, the interpretation of enantiomeric composition must be done cautiously. Many compounds will undergo racemization under conditions of low pH and/or high temperature which can occur during storage, processing, sample preparation or analysis. Therefore, further work is needed to establish reliable criteria for distinguishing natural and synthetic flavorings in foods and beverages. Also, as cautioned by Mussinan and Hoffman (7), “naturalness” cannot be unambiguously assigned if the compound is obtained from a previously unknown natural process or source. References of known chirality are also needed in order to definitively confirm enantiomeric structure; these references are frequently not commercially available and must be purified in the analyst’s lab.

Finally, other precautions are becoming necessary as biomimetic syntheses become more common. Sewenig et al. (15) recently showed that enantioselective synthesis of (R)- α -ionone was possible from racemic (“synthetic”) precursors and therefore the complete authenticity of raspberry flavorings was only possible when enantioselective gas chromatography was combined with isotope ratio mass spectrometry to provide accurate and precise measurements of $^2\text{H}/^1\text{H}$ and $^{13}\text{C}/^{12}\text{C}$ isotope ratios.

Species, Varietal, or Cultivar Characterization

Along with the recognition that biological processes often produce specific enantiomeric ratios of different compounds, is the fact that different species, varieties, and cultivars may exploit different enzymatic or biosynthetic pathways

resulting in different final product compositions. For example, Engel and co-workers observed that the enantiomeric composition of the alkan-2-ols, alkan-2-yl esters, and 3-hydroxyacid esters were different in yellow and purple passion fruit (*Passiflora edulis* f. *flavicarpa* and *Passiflora edulis* Sims, respectively) (16-18). They proposed that different enzymes might be involved in the reduction of a methyl ketone precursor leading to (S)(+)-secondary alcohols in the case of yellow passion fruit and (R)(-)-alcohols in purple passion fruit (16).

Guichard (19) studied the chiral lactones in apricot and showed that the R-isomer of the C6, C7, C8, C9, and C12 gamma-lactones were predominant. Guichard proposed that this information could be used to confirm "natural" apricot flavors. However, an evaluation of the lactone R/S ratio in a variety of apricot cultivars indicated that for some of the lactones, the R/S ratio remained constant among cultivars (e.g., C8), while for other lactones, the R/S ratio was variable among cultivars (e.g., C6, C9) (Table 1). Although not explicitly discussed in this paper, it appears that these ratios could be used to identify and/or confirm the identity of the various cultivars.

Table I. Chiral Gamma-Lactones in Apricot Cultivars

<i>Cultivar</i>	<i>R/S Ratio of γ-Lactones</i>					
	<i>C6</i>	<i>C7</i>	<i>C8</i>	<i>C9</i>	<i>C10</i>	<i>C12</i>
PT	99	81	90	64	91	93
PA	82	80	90	--	92	99
MO	91	81	90	83	95	99
RR	71	90	88	77	98	99
PO	96	81	92	76	95	99
BE	88	89	93	83	96	98

Adapted from reference 19.

In another study, Mosandl et al. (13) showed that different species of mint could be identified based on enantiomeric ratios of three terpenes, α -pinene, β -pinene, and limonene, in the oil. Engel and co-workers were able to distinguish different corn varieties by the chiral composition of the secondary alcohols (17, 18). In blackcurrants, Ruiz del Castillo and Dobson (20) observed that the terpene alcohols, terpinen-4-ol and linalool, displayed large differences in

enantiomeric composition among cultivars, even though the composition of many other monoterpenes was not greatly different. Finally, the 3-butylhexahydrophthalide stereoisomers were used to distinguish among different *Apium graveolens* L. varieties (celery and celeriac) (21).

Use of enantiomeric ratios for authentication studies requires that the chiral composition remain constant during ripening and maturation of the plant tissues. For example, the enantiomeric ratios of hydroxy- and acetoxyacid esters in pineapple and lactones in nectarines and peaches did not change during maturation even though total concentrations of these compounds increased (17, 18, 22). However, for many products, changes in enantiomeric composition during maturation are not known. In addition, care must be taken that the same tissue type is used for comparison in these types of studies. For example, Bartschat et al. (21) showed that celery (*Apium graveolens*) seed had different ratios of the 3-butylhexahydrophthalide stereoisomers than did the stalk and root.

In authentication studies of this type, further understanding of the mechanisms and precursors involved in the biosynthetic pathways are also needed. In conjunction with increased knowledge of genetic sequence information for many plants, different species, varieties, and/or cultivars may be best differentiated in the future by a combination of genetic, proteomic (enzymatic), and metabolomic information.

Determination of Geographic Origin

Use of enantiomeric analysis to confirm point-of-origin has been more limited. König et al. (4) observed that different ratios of (+/-)-borneol were present in rosemary oil depending on whether the oil was sourced from Tunisia or Spain. When combined with other differences in volatile composition, these authors indicated that chiral composition could provide country-of-origin information. In another study, differences in stereoisomeric theaspiranes and theaspirones were found in raspberries obtained from France or Yugoslavia (23). Werkhoff et al. (24) also observed differences in enantiomeric composition of γ -decalactone isomers in raspberry samples from different countries-of-origin.

These and related studies are limited however by the fact that in most cases no varietal information was available and no information on processing conditions or product maturity was provided. This again points to the need for careful control and verification of these variables in authentication studies. Even in instances where these variables are thought to be controlled, it is possible that misidentification can occur as indicated by the relatively common misidentification of grape cultivars in many vineyards (25, 26) (see also chapter by von Baer et al.).

Confirmation of Processing/Aging

Frequently, enantiomeric composition will change during food and beverage processing, and these changes may provide a useful tool for monitoring or confirming various processing treatments. For example, Pierce et al. (27) proposed that thermal processing of cherries could be monitored by determining the racemization of limonene, linalool and α -terpineol during heating. Güntert et al. (28) observed that the enantiomeric excess of the compound filbertone ((E)-5-methyl-2-hepten-4-one) in hazelnuts (*Corylus avellana*) changed during the roasting process. In this case, racemization during roasting was not thought to be responsible for the change in enantiomeric composition since pure (E, S)-filbertone was not sensitive to heat. The authors hypothesized that filbertone may be released from an unidentified precursor during heating, producing racemic filbertone by a purely chemical mechanism.

Many bacterial fermentation processes result in formation of unique enantiomeric profiles which may be used to confirm microbial processing, contamination, or spoilage. For example, D-amino acids are common in most fermented foods such as cheese, yoghurt, wine, and vinegar (reviewed by 2). Products which have not undergone microbial fermentation, such as 'sour cream' produced by addition of acid rather than with microbial cultures, would not exhibit this characteristic enantiomeric profile. In distilled beverages, the stereochemical analysis of 2-butanol and its precursor 2,3-butanediol in alcoholic beverages has been used to determine the presence of spoilage bacteria during fermentation or distillation (29-32).

The natural racemization of chiral compounds has also been explored to study wine aging. Full and Winterhalter (33) observed that changes in the ratios of isomeric vitispiranes which occurred during aging (Figure 1) may be used to determine or confirm the age of bottled Riesling wines. The wines in this study were purchased commercially from a number of producers and regions.

In another study, Calabrese et al. (34) observed that the amount of D-proline increased during wine aging and they proposed that the D/L ratio could be used as an indicator of wine age (Figure 2). Calabrese et al. (34) used a number of different red and white wine varieties which were obtained from several regions and manufacturers in Italy.

During distribution and transport it is possible for wines to be exposed to relatively high temperatures for extended time periods which may alter enantiomeric composition. In the studies of Full and Winterhalter (33) and Calabrese et al. (34), the effect that elevated storage and processing temperatures would have had on the enantiomeric ratios is unknown, and again may be a limitation of the use of enantiomeric composition alone as a marker for wine age.

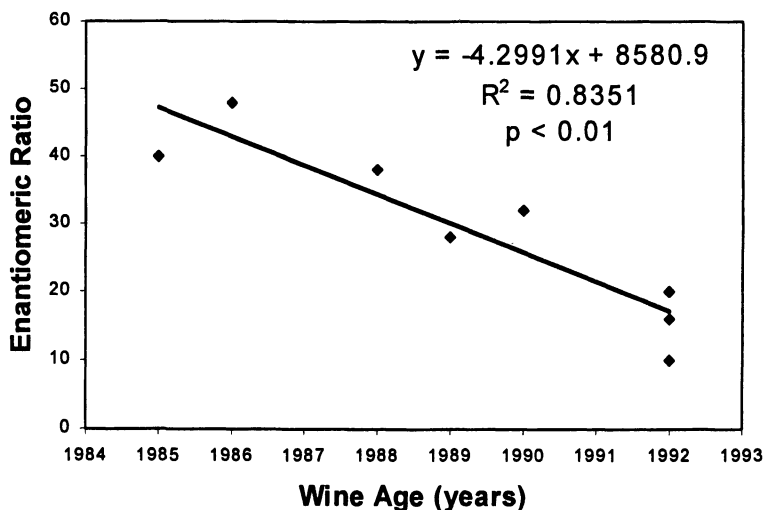


Figure 1. Isomeric vitispiranes in aged White Riesling wines. Enantiomeric excess of 2R, 5S relative to 2S, 5R is shown. Adapted from reference 33.

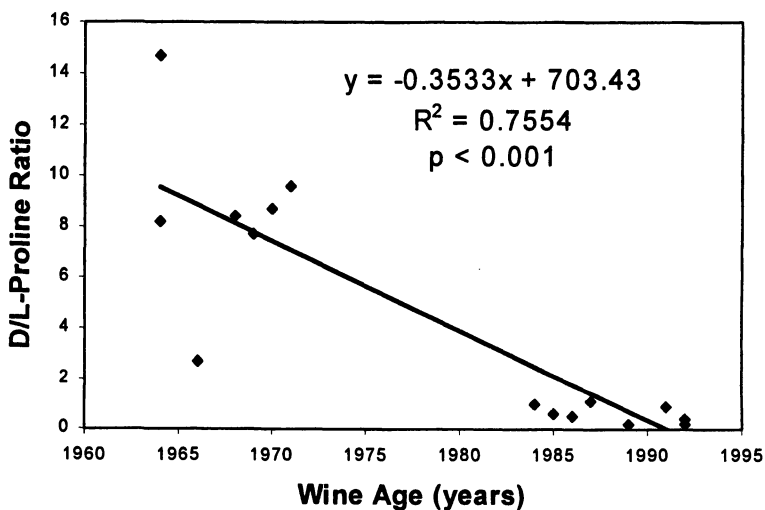


Figure 2. Changes in ratio of D- and L-proline during wine aging. Adapted from reference 34.

Summary

As chemists become increasingly sophisticated in their ability to mimic "natural" processes in the laboratory, analysts and regulatory agencies will need to develop increasingly sophisticated tools for comprehensive authenticity assessments. Chiral analysis has been a valuable tool for determining naturalness of flavors and essential oils, but in the future, will need to be combined with multiple analytical tools (e.g., isotope ratio mass spectrometry and NMR) for complete authentication. In all cases, further studies are needed to evaluate the variations in chemical composition, including variations in enantiomeric composition, with respect to thermal processing, pH conditions, product maturity or age, geographic point-of-origin, and varietal or cultivar variations. These studies will require advances in analysis speed in order to efficiently analyze large numbers of samples, the ability to process large amounts of data, and multivariate statistical tools in order to fully understand the effects of multiple variables on a wide range of chemical compounds present in foods and beverages. Finally, complementary information from genetic, proteomic, and metabolomic sources may be required for future authentication of food and beverage products.

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Chapter 4

Sophisticated Online Techniques in the Authenticity Assessment of Natural Flavors

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The acceptance of food strongly depends on their flavor and aroma impressions. Consequently, authentication of genuine flavors is an important topic in view of quality assurance in the food industry and in consumer protection. Both phenomena, *enantioselectivity* as well as *isotope discrimination* during biosynthesis, may serve as inherent parameters in the authenticity control of natural flavor compounds, provided that suitable methods and comprehensive data from authentic sources are available. Besides site specific *natural isotope fractionation*, measured by NMR-spectroscopy (SNIF-NMR), enantio-selective *capillary gas chromatography* (enantio-cGC) and comparative *isotope ratio mass spectrometry* (IRMS), have proved to be highly efficient tools in the origin specific analysis. Nevertheless, analytical authentication of genuine food constituents is a permanent challenge, due to the complexity of food matrices. So far, enantioselective and/or IRMS online coupling techniques are the methods of choice in order to determine the authenticity of flavors. The benefits of *stir bar sorptive extraction* (SBSE)-enantio-MDGC/MS and multielement GC-IRMS techniques are outlined.

Stir Bar Sorptive Extraction (SBSE)-enantio-MDGC-MS

A novel, solventless, and simple technique for extraction of organic analytes from aqueous samples [stir bar sorptive extraction (SBSE)], has been recently introduced (1). SBSE takes advantage of the high enrichment factors of sorptive beds, but with the application range and simplicity of SPME (2-5). The stir bar is coated with a thick film of polydimethylsiloxane (PDMS), in which the aqueous sample extraction takes place during stirring for a predetermined time. After that time it is removed and placed into a glass tube, which is transferred into a thermal desorption system (TDS) where the analytes are thermally recovered, cryofocused and evaluated on-line with a capillary MDGC-MS system (Figure 1). In addition to the extraction of organic analytes from aqueous samples, the PDMS stir bars are also suitable for headspace and *in vivo* headspace sampling. Headspace sampling is a technique widely used to characterize the volatile fraction of several matrices, particularly aromatic and medicinal plants. SBSE has also been shown to be a successful technique for headspace sampling, because the PDMS stir bars enrich higher amounts of trapping material than SPME and, therefore, exhibit better extraction efficiency for analyzing minor components (6). Enantioselective analysis has been widely applied in quality assurance of natural flavors and essential oils, and the knowledge of enantiomeric ratios of characteristic constituents can be used as a potent genus chemitaxonomic marker. The enantioselective analysis of chiral compounds in complex natural materials by multidimensional gas chromatography (MDGC) has been shown to be an efficient and selective tool for this purpose (7-9) This connection allows the combination of the high extraction efficiency of the stir bar with the high selectivity of the enantio-MDGC-MS system. Thus, it is possible to determine the exact enantiomeric ratios of minor components in complex natural materials such as food flavors or essential oils.

SBSE Sampling

A stir bar consisting of a magnetic core sealed inside a glass tube with a length of 1.2 cm, an outer diameter (o.d.) of 1.2 mm and coated with 55 μ L PDMS was used. Stir bars are manufactured and offered by Gerstel (Mühlheim/Ruhr, Germany) under the name Twister[®]. The stir bar is conditioned in a desorption tube (178 mm length, 6 mm o.d., 4 mm i.d. glass tube) of a thermal desorption unit (Gerstel TDS-2) at 300 °C for 2 h.

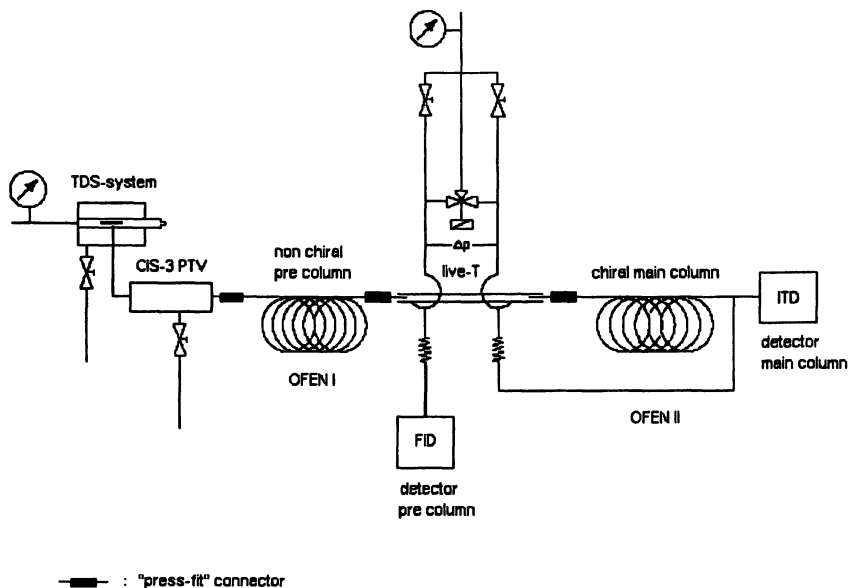


Figure 1. Schematic diagram of SBSE-enantio-MDGC-MS
(Reproduced from reference 10.)

Essential Oil Sampling Procedure

The conditioned stir bar is placed in a 10 mL water sample with an essential oil concentration of 1 ppm and stirred at 1200 rpm for 20 min at ambient temperature. The stir bar is removed and dried, using a clean paper cloth. After positioning the stir bar in the middle of the heating zone of the desorption tube, the desorption program is started.

Headspace Stir Bar Sorptive Extraction (HSSE)

Essential oil vapors of 0.3 g carefully dried plant material of *Melaleuca alternifolia* (C.), *Eucalyptus globulus* (L.) and *Thymus vulgaris* (L.) are extracted by a Twister[®] coated with 55 μ L film of polydimethylsiloxane, which is held at one end of magnetic wire fastened at the lid of a 20 mL vial. After an equilibration time of 15 min the stir bar is removed and positioned in the middle of the heating zone of the desorption tube and the desorption program is started. The headspace sampling device is outlined in Figure 2.

In Vivo-HSSE

A stir bar was placed in a 25 mL flask, which was fixed to a holder near the living plants and contained two or three branches. The opening of the flask was sealed carefully with Parafilm® and, after an equilibration time of 15 min for *Melaleuca alternifolia* (C.), 30 min for *Eucalyptus globulus* (L.) and *Thymus vulgaris* (L.), the stir bar was removed and the analytes were desorped as described before. The *in vivo* headspace sampling device is outlined in Figure 2.

Thermal Desorption—Capillary MDGC—MS

The TD—MDGC—MS consists of a Gerstel TDS thermal desorption system mounted on a Siemens Sichromat 2, with two independent column oven programs and a live T-switching device, coupled to the transfer line of a Finnigan MAT ITD 800, using an open split interface. For thermal desorption conditions, cf. ref. (10).

Results and Discussion

In order to obtain accurate information about the enantiomeric purity of chiral compounds in essential oils, analytical procedures of highest selectivity are mandatory. Principal limitations of enantioselective analyses are known, such as racemates of natural origin, generated by non-enzymatic reactions (autoxidation, photooxidation, etc.), racemization during processing or storage (if structural features of chiral compounds are sensitive) and blending of natural and synthetic chiral compounds (9).

A typical well-known example is the acid-catalyzed racemization of linalool during distillation (11-13). The pH value of plant material and distillation time are critical parameters in the chirality evaluation of pH-sensitive compounds. One possibility for obtaining accurate information about the native enantiomeric purity of labile compounds is to avoid the racemization or formation of artifacts during processing by using the headspace analysis of plant material. HSSE—enantio-MDGC—MS is a new method for the reliable determination of genuine enantiomeric ratios, as well as the *in vivo* HSSE technique. The enantiomeric purities of chiral monoterpenes in the gaseous emission of the living plants *Melaleuca alternifolia* (C.), *Eucalyptus globulus* (L.) and *Thymus vulgaris* (L.) are determined and compared with the results of the headspace sampling and essential oil analysis of authentic materials.

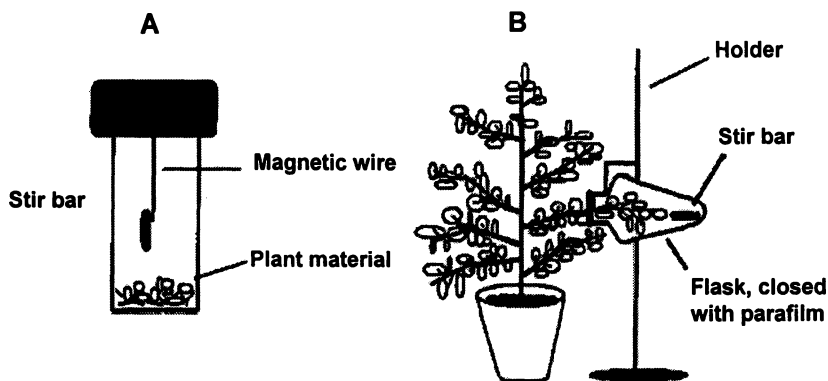


Figure 2. Scheme of the headspace sampling (A), in vivo headspace sampling device (B). (Reproduced from reference 10.)

Figure 3 shows the simultaneous enantioselective analysis of the standard compounds α -pinene (1), β -pinene (3), α -phellandrene (4), linalool (6), terpinen-4-ol (7), α -terpineol (9), borneol (8), sabinene (2) and limonene (5), experimental data see (10).

Conclusion

The combination of SBSE, thermal desorption and enantio MDGC-MS allows the determination of the enantiomeric purity of chiral flavor compounds in essential oils and fruits, avoiding the usual expensive and time consuming isolation steps for complex matrices. The presented sampling methods are suitable for establishing characteristic authenticity profiles of fruit flavors by enantioselective capillary gas chromatography and may be utilized in quality assurance of processed fruits (Table I).

Isotope Discrimination

The natural cycles of the bioelements carbon, oxygen, hydrogen (nitrogen, sulfur) are subjected to various discrimination effects, such as thermodynamic isotope effects during water evaporation and condensation or isotope equilibration between water and CO_2 . On the other hand the processes of photosynthesis and secondary plant metabolism are characterized by kinetic isotope effects, caused by defined enzyme catalyzed reactions (15).

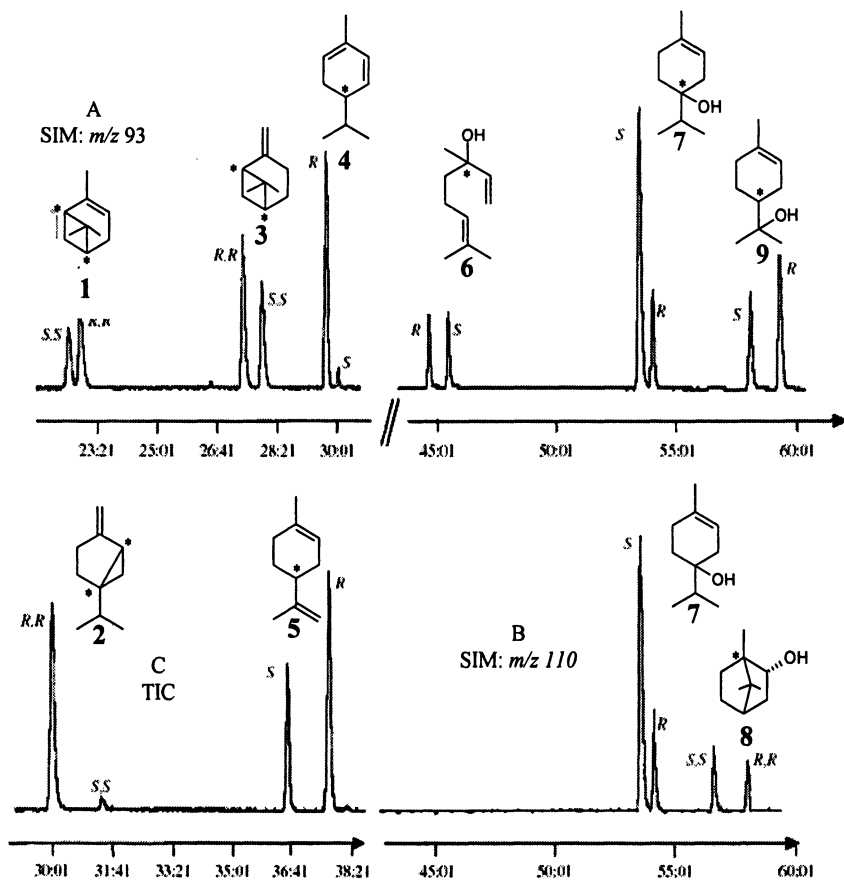









Figure 3. SBSE-enantio-MDGC analysis of the standard compounds α -pinene (**1**), sabinene (**2**), β -pinene (**3**), α -phellandrene (**4**), limonene (**5**), linalool (**6**), terpinen-4-ol (**7**), borneol (**8**), α -terpineol (**9**), main column; A and B, temperature program 1; C, temperature program 2. (Reproduced from reference 10.)

Table I. Enantiomeric purity [%] of fresh Andalusian strawberries and products, using SBSE-*enantio*-MDGC/MS

							
	1	2	3	4	5	6	7
pulp	R: >99 S: >99	R: 1,5 S: 98,5	R: n.d. S: n.d.	R: >99 S: <1	S: <1 R: >99	R: 97,0 S: 3,0	R: >99 S: <1
jam	R: <1 S: >99	R: <1 S: >99	R: n.d. S: n.d.	R: >99 S: <1	S: <1 R: >99	R: 97,1 S: 2,9	R: >99 S: <1
in fruit	R: 1,4 S: 98,6	R: <1 S: >99	R: n.d. S: n.d.	R: n.d. S: n.d.	S: <1 R: >99	R: 97,5 S: 2,5	R: >99 S: <1
head-space	R: 1,9 S: 98,1	R: <1 S: >99	R: n.d. S: n.d.	R: n.d. S: n.d.	S: <1 R: >99	R: >99 S: <1	R: >99 S: <1

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The highly precise measurement of isotope ratios has a long tradition in organic geochemistry. Nowadays, the elucidation of stable isotope distributions is highly desirable in view of fundamental studies in biochemistry, nutrition, drug research and also in the authenticity assessment of food ingredients.

In 1981 G. J. Martin and M. L. Martin (16) showed that the ^2H distribution of organic molecules does not follow a statistic pattern, but is discriminated by isotopic effects, measurable by ^2H -NMR and IRMS respectively. Meanwhile, systematics of $^{18}\text{O} / ^2\text{H}$ patterns in natural plant products are more and more understood and reported by Schmidt et al. (17-19) as new and reliable tools for the elucidation of biosynthetic pathways and helpful indicators in the authenticity assessment of natural compounds.

Stable isotope ratio analysis, measured by ^2H -site-specific nuclear magnetic resonance (^2H -SNIF®-NMR) and $^{18}\text{O}/^{16}\text{O}$ isotope ratio mass spectrometry (IRMS) have been adopted as official methods by the Commission of the European Communities (EC). These methods play a key role in detecting adulterations like addition of water and inadmissible wine sweetening or chaptalization with beet or cane sugar (20).

GC- IRMS techniques

Fundamentals

Isotope ratio mass spectrometry (IRMS) has become more and more important in food authenticity assessment, since capillary gas chromatography (cGC), coupled on-line via a suitable combustion- / pyrolysis interface with IRMS, has been realized. The substances eluting from the cGC column are converted into the corresponding gas (carbon dioxide, nitrogen, hydrogen and carbon monoxide, respectively) and then directly analyzed in the isotope mass spectrometer (Table II, III). The spectrometer is adjusted for the simultaneous recording of the reactand gas isotopomers. Thus, the components can be detected in the nmole range with high precision.

Table II. IRMS-Online Coupling Techniques

GC-combustion-IRMS (GC-C-IRMS)	$\delta^{13}\text{C}$
GC-combustion/reduction-IRMS	$\delta^{15}\text{N}$
GC-pyrolysis-IRMS (GC-P-IRMS)	$\delta^{18}\text{O}$
	$\delta^2\text{H}$
Thermochemical conversion/element analyzer (TC/EA)	$\delta^{18}\text{O}$
	$\delta^2\text{H}$

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Table III. Specifications for cGC-IRMS Coupling Techniques using DELTAplus XL, Thermo Electron, Bremen (Germany)

Bioelement	Analyzed Gas	On column		
		Need (mol)	Need (ng)	Precision
Carbon	CO ₂	0.8 nmol C	10 ng C	0.2 per mil
Nitrogen	N ₂	1.5 nmol N ₂	42 ng N	0.5 per mil
Hydrogen	H ₂	15 nmol H ₂	30 ng H	3.0 per mil
Oxygen	CO	5 nmol O	80 ng O	0.8 per mil

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Validation

Isotope ratios are given as deviations, in relation to a defined primary standard (zero point). The polyethylene foil CH_7 or NBS-oil are commercially available secondary standards, certificated and managed by the *International Atomic Energy Agency* (IAEA). However, up to now GC-IRMS systems can not be calibrated without the aid of alternative peripheries like elemental analyzer (EA) or dual inlet, due to the lack of commonly accepted reference material, applicable in GC-IRMS techniques (Figure 4).

In the course of a feasibility study, sponsored by the European Union, the components of the *GC separation efficiency test*, according to K. Grob were tested in their usability as certificated tertiary standards. Seven compounds are now available as a ready-to-use mixture for testing the accuracy of the GC-IRMS measurements, and furthermore, simultaneously provides important information about the actual quality status of the GC-column system used (22).

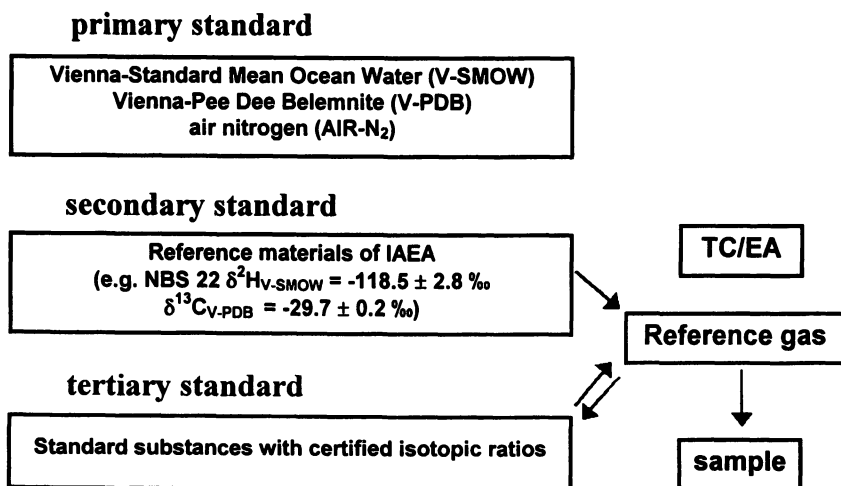


Figure 4. GC-IRMS – calibration of the reference gas.
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The isotope ratio traces of the GC peaks exhibit a typical S-shape. The heavier isotopic species of a compound elute more rapidly than the light species. Similar effects can be observed for all chromatographic processes, whereas the size of isotope fractionation and the elution order of the isotopomers depends on: (1) the chromatographic system applied, (2) the temperature, and (3) the structural features of the compounds analyzed. In any case care must be taken to integrate across the full width of the chromatographic peaks. Of course, reliable results on isotopic ratios from cGC-IRMS experiments can only be expected from very high-resolution cGC ($R_s \geq 1.5$). Also, accurate sample clean-up procedures without any isotope fractionation must be guaranteed. Under the conditions of validated procedures and calibrated instruments IRMS-data are valuable indicators in the authenticity assessment of flavor and fragrance compounds.

As the latest development MDGC is reported, online coupled with IRMS. This coupling technique combines the advantages of both highly sophisticated techniques, to achieve the utmost accuracy of IRMS measurements (23).

In deed, MDGC-IRMS is the method of choice for precise and accurate measurements of compounds from complex matrices, under the condition, that the analyte is quantitatively transferred from the pre column eluate to the main column.

Comprehensive authenticity assessment

Lavender oil

For hundreds of years the essential oil of lavender has been well appreciated for perfumery purposes (24). Lavender oil is obtained by steam distillation from the fresh flowering tops of *Lavandula angustifolia* Miller (*Lavandula officinalis* Chaix) (25). It is a colorless or pale yellow, clear liquid, with a fresh, sweet, floral, herbaceous odor on a woody balsamic base (25, 26). According to the European Pharmacopoeia, characteristic components of lavender oils are limonene, cineol, 3-octanone, camphor, linalool, linalyl acetate, terpinen-4-ol, lavandulyl acetate, lavandulol and α -terpineol. Adulterations commonly include blends of lavender oils with lavandin oil or spike oil, and the addition of synthetic linalool and linalyl acetate. In contrast, genuine lavender oils contain as main constituents (R)-linalyl acetate and (R)-linalool of high enantiomeric purity. For that reason enantioselective analysis of linalool and linalyl acetate was proved to be a powerful tool to detect adulterations with synthetic racemates

of linalool and linalyl acetate respectively (27, 28). To conclude from the latest documentation of the European Directorate for the Quality of Medicines, European Pharmacopoeia Commission, the enantiomeric purity of linalool and linalyl acetate has been adopted into monograph no. 1338 Lavender oil of European Pharmacopoeia. In accordance with this documentation the percentage content of linalool (20.0–45.0%) and linalyl acetate (25.0–46.0%) in conjunction with the specification of (S)-linalool (maximum 12%) and (S)-linalyl acetate (maximum 1%) is now defined as a concept for the authenticity assessment of lavender oil (25). However, by using upcoming techniques like simulated moving bed chromatography (29), the generation of large amounts of enantiopure (R)-linalool from synthetic racemate has become realistic. Consequently, enantioselective analysis may no longer be sufficient for the unambiguous authenticity assessment of lavender oils (21).

The determination of $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$ values, of synthetic and natural linalool and linalyl acetate using isotope ratio mass spectrometry (IRMS) has been reported by different authors (12, 17, 30-33). Using a pyrolysis interface the determination of $^{18}\text{O}/^{16}\text{O}$ isotope ratios has been proved to be a further useful indicator in the authenticity assessment of lavender oils. Thus, self-prepared essential oils of fresh lavender by means of steam distillation are used for determining the natural range of $\delta^{18}\text{O}_{\text{V-SMOW}}$, $\delta^2\text{H}_{\text{V-SMOW}}$ and $\delta^{13}\text{C}_{\text{V-PDB}}$ values of linalool and linalyl acetate.

In terms of authenticity assessment three-dimensional plots of the $\delta^{18}\text{O}_{\text{V-SMOW}}$, $\delta^2\text{H}_{\text{V-SMOW}}$ and $\delta^{13}\text{C}_{\text{V-PDB}}$ values are presented for both linalool and linalyl acetate.

The plot of linalool shows five commercial samples S1–S5(Ⓢ) clearly differentiated from those of authentic sources and from all the other oils and standards analyzed (Figure 5). It is obvious that samples S1–S5 show higher linalool $\delta^2\text{H}_{\text{V-SMOW}}$ and $\delta^{18}\text{O}_{\text{V-SMOW}}$ values than all the other samples. On the other hand, as to conclude from their enantiomeric ratios samples S1–S5 must be interpreted as blends with more or less synthetic linalool and linalyl acetate respectively (Table IV). The linalyl acetate plot (Figure 5) shows similar results for the commercial samples S1–S5, which differ from the authentic samples as in the linalool plot (Figure 5). These five oils are clearly out of the authenticity range and have to be classified as nonauthentic samples. In order to put the isotopic data of linalool and linalyl acetate into one three-dimensional plot and to emphasize the results presented, the differences of the stable isotope ratios of linalool and linalyl acetate are depicted as a three-dimensional plot of Δ values (δ values of linalool minus δ values of linalyl acetate for oxygen, hydrogen and carbon) (Figure 6). This plot shows that the commercial samples S1–S5 are different from all the other samples investigated. Linalool and linalyl acetate of S1–S5 definitely are not genuine lavender oil compounds. Furthermore, it is

noteworthy that two commercial samples (A1, A2) differ from other commercial oils and from self-prepared oils owing to their $\delta^{13}\text{C}_{\text{V-PDB}}$ values of linalool, but there is no significant difference concerning oxygen and hydrogen stable isotope ratios. However, when the isotopic ratios of oxygen, hydrogen and carbon from the corresponding linalyl acetate are considered, samples A1 and A2 are completely inconspicuous with respect to the other commercial oils investigated (Figure 5). A reliable authenticity assessment is concluded from the simultaneous consideration of multielement IRMS and enantioselective analysis for the following reason: the $\Delta^{13}\text{C}_{\text{V-PDB}}$ values ($\delta^{13}\text{C}_{\text{V-PDB}}$ linalool $\delta^{13}\text{C}_{\text{V-PDB}}$ linalyl acetate) of A1 and A2 significantly differ from those of authentic samples. According to Kreis and Mosandl (27) high enantiomeric purities of (R)-linalool (above 94 %) and (R)-linalyl acetate (above 98%) are known as characteristics of authentic lavender oils. In samples A1 and A2 the enantiomeric purity of (R)-linalyl acetate is better than ever detected in authentic lavender oils [above 99.9% (R)-enantiomer], whilst (R)-linalool is detected at the lower range of admissible purity of genuine linalool from lavender, produced under good manufacturing practice conditions [96.2 and 95.9% (R)-linalool, respectively] (Table IV). Consequently, considering $\Delta^{13}\text{C}_{\text{V-PDB}}$ values in conjunction with enantio-MDGC-MS analysis, leads to the conclusion that linalool and linalyl acetate from samples A1 and A2 do not originate from lavender (Figures 5, 6, Table IV).

Table IV. Enantiomeric ratios of linalool and linalyl acetate from nonauthentic samples.

Sample	Linalool		Linalyl acetate	
	(R)	(S)	(R)	(S)
A1	96.2	3.8	>99.9	<0.1
A2	95.9	4.1	>99.9	<0.1
S1	70.7	29.3	52.9	47.1
S2	55.5	45.5	55.7	44.3
S3	62.0	38.0	51.8	48.2
S4	69.7	30.3	52.0	48.0
S5	60.8	39.2	53.3	46.7

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(E)- α (β)-Ionone

The on-line determination of $\delta^2\text{H}_{\text{V-SMOW}}$ values using gas chromatography-pyrolysis-isotope ratio mass spectrometry (GC-P-IRMS) has been developed recently (35) and has proved to be a powerful tool to define the authenticity of natural compounds (28, 36-39). However, as fruit flavor extracts are rather complex, and the sample amount for hydrogen measurement has to be

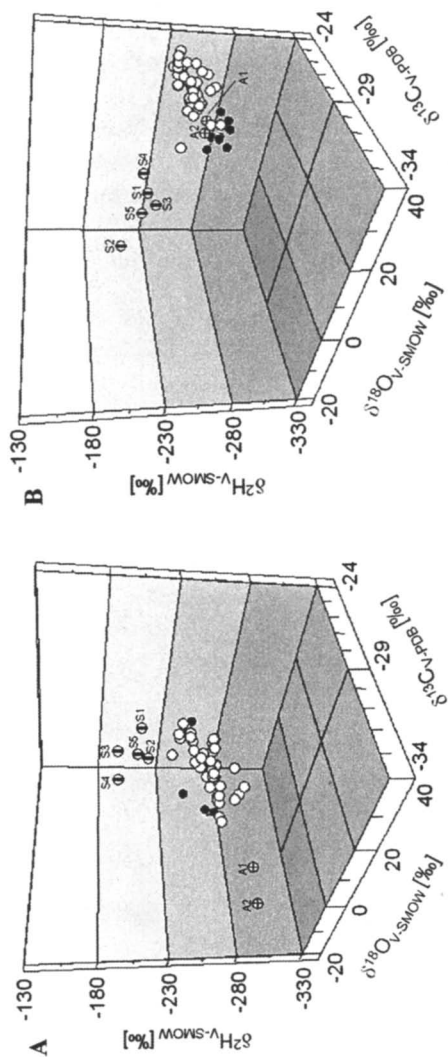


Figure 5. (A) Multielement-IRMS-analysis of linalool from different origins: authentic (●), commercial (○), commercial non-authentic (⊖) and special aberrations (⊕) (B) Multielement-IRMS-analysis of linalyl acetate from different origins: authentic (●), commercial (○), commercial non-authentic (⊖) and special aberrations (⊕). (Reproduced from reference 34.)

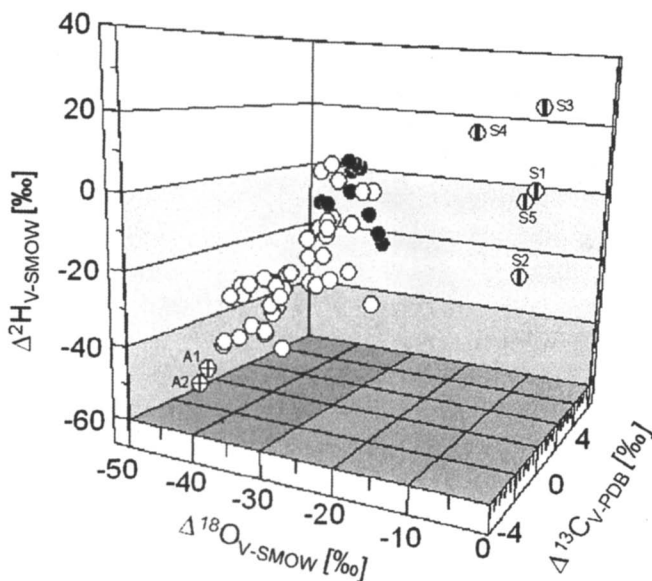


Figure 6. Multielement-IRMS-analysis of lavender oil main compounds. Differential diagram ($\Delta = \delta_{\text{linalool}} - \delta_{\text{linalyl acetate}}$); authentic (●) and commercial (○) samples; commercial non-authentic (⊕) and special aberrations (⊕). (Reproduced from reference 34.)

rather high due to the low abundance of deuterium isotopes, the use of single GC-IRMS is often not sufficient for the precise and accurate $\delta^2\text{H}$ measurements of characteristic aroma components from fruit flavor extracts. MDGC-IRMS technique was developed and introduced to the practice of authentication by Juchelka et al. (23) and Asche et al. (40), but until now this technique has been applicable only in the determination of $^{13}\text{C}/^{12}\text{C}$ ratios, for the following reason: As the carrier gas flow strongly depends on temperature, the classical pressure-controlled column-switching technique, which was introduced by Deans in 1968 and realized in a modified version within the Siemens Sichromat MDGC system (41), is unsuitable in evaluating $^2\text{H}/^1\text{H}$ isotope ratios, when temperature programmed column switching becomes necessary.

The importance of a constant carrier gas flow to accurate $^2\text{H}/^1\text{H}$ isotope ratio measurements was demonstrated by Bilke et al. (42). A suitable residence time in the reactor is mandatory for a complete and subsequently quantitative pyrolysis, free of isotope discrimination. Furthermore, the sample amount reaching the reactor is flow-dependent. With higher column temperature and constant gas pressure the carrier gas flow decreases and less sample will pass the reactor in a certain time interval. This is why the constant-flow MDGC

option was recognized as an essential prerequisite of reliable $\delta^2\text{H}$ measurements. To meet these requirements, the Multi Column Switching System MCS 2 was used. The accuracy and precision of this column-coupling technique is proved by comparative standard measuring using TC/EA-IRMS and MDGC-P-IRMS (Table V). Subsequently the isotopic ratios of the analytes (*E*)- α (β)-ionone from raspberry fruits and raspberry products available on the market were determined.

(*E*)- α -Ionone is known to be an important constituent in several aroma extracts from black tea, violet flowers, vanilla pods, *Osmanthus*, and *Saussurea lappa* Clarke (costus root), and both (*E*)- α - and (*E*)- β -ionone can be found in carrots, *Boronia megastigma*, and raspberry fruits. The (*R*)-enantiomer of (*E*)- α -ionone is detected with high enantiomeric purity ($>99\%$). Hence, the authenticity of (*E*)- α -ionone is mostly proved via enantio-GC applications (43-46). In the majority of cases synthetic ionones are produced via pseudoionone, prepared by base-catalyzed condensation of citral with acetone. After acidic catalysis (using 85% phosphoric acid or concentrated sulfuric acid), this reaction yields racemic (*E*)- α -ionone and (*E*)- β -ionone (47).

With new upcoming techniques, such as simulated moving bed (SMB) chromatography (29), the production of large amounts of enantiopure (*R*)-configured (*E*)- α -ionone from synthetic (*E*)- α -ionone racemate is conceivable as reported by Morbidelli et al. (48). Consequently, enantioselective analysis is no longer sufficient for a comprehensive authenticity assessment of the named extracts (21) and, in general, the use of multielement/multicomponent IRMS analysis - in addition to enantioselective capillary GC - becomes more and more important. Constant flow multidimensional gas chromatography-combustion/pyrolysis-isotope ratio mass spectrometry (MDGC-C/P-IRMS) and enantioselective MDGC analysis are proved to be the most efficient on-line coupling techniques in the direct and comprehensive authenticity assessment of chiral and nonchiral analytes, such as (*E*)- α -ionone and (*E*)- β -ionone, from complex matrices without any risk of discrimination (49).

Results And Discussion

In Figure 7 a schematic diagram of the new MDGC-P-IRMS system is shown. The precolumn and main column are connected via the multicolumn switching system MCS2, which is located in the precolumn oven. The precolumn retention times of the interesting compounds are determined by a monitor detector; hence,

Table V. Comparison of $\delta^2\text{H}_{\text{V-SMOW}}$ values of tertiary standards, measured by TC/Ea-IRMS and MDGC-P-IRMS

	TC/Ea-IRMS		MDGC-P-IRMS		
	mean (‰)	n ^a	mean (‰)	σ^b (‰)	Δ (MDGC-TC/Ea) (‰)
5-nonanone	-89±3	30	-88	1.0	1
linalool	-190±4	30	-190	2.3	0
(-)-menthol	-242±3	30	-239	1.4	3
linalyl acetate	-181±4	30	-184	2.0	-3
γ -decalacton	-191±3	30	-191	1.2	0
(E)- α -ionone	-197±3	30	-196	1.8	1
1-octanol	-68±2	10	-72	1.4	-4
dodecane	-128±3	10	-127	1.4	1
methyl decanoate	-246±2	10	-247	0.8	-1
methyl <i>N</i> -methylanthranilate	-133±4	10	-127	0.6	6
methyl dodecanoate	-250±3	10	-249	1.8	1

^aNumber of measurements. ^bStandard deviation.

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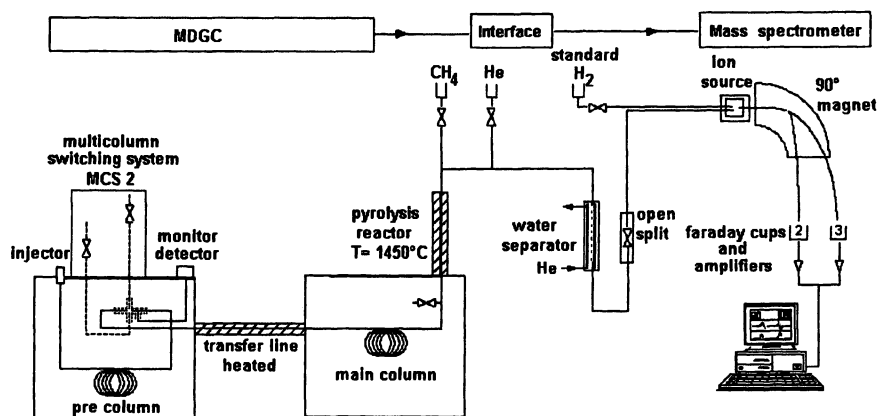


Figure 7. Schematic diagram of MDGC-pyrolysis-IRMS. (Reproduced from reference 49. Copyright 2005 American Chemical Society.)

the right cut times for the transfer onto the main column can be established. Cutting is realized by different gas flows through the MCS2 device; there are two adjustments: first, the effluent from the precolumn is faded-out by a counter current flow after passing the precolumn detector (*cut on*); second, the precolumn effluent reaches the main column, when the counter current flow is switched off (*cut off*).

By measuring standard compounds [5-nonanone, linalool, (-)-menthol, linalyl acetate, γ -decalactone, (*E*)- α -ionone, 1-octanol, dodecane, methyl decanoate, methyl dodecanoate, and methyl *N*-methylantranilate], comparatively with TC/EA-IRMS and MDGC-P-IRMS, the accuracy of the new method was successfully demonstrated. As summarized in Table V all values determined via MDGC-P-IRMS comply with the TC/EA-IRMS values within the standard deviation range of 0-6%. Thus, the direct and nonisotopic discriminating sample preparation via MDGC is proved. Of course, quantitative transfer of the substances is mandatory to obtain accurate isotopic values (23). To point out the relevance of the new coupling technique, Figure 8 shows a precolumn (A) and a main column (B) chromatogram of a raspberry extract (variety Rucami) measured by MDGC-P-IRMS. The concentrations of (*E*)- α -ionone and (*E*)- β -ionone are adjusted to the linearity range of the IRMS (peak amplitude: 4-7 V).

It is obvious that the precolumn separation of (*E*)- α -ionone is not sufficient for precise isotopic measurements. However, by cutting exclusively the precolumn section of (*E*)- α (β)-ionone onto the main column, a sufficient chemical purification and adequate performance are achieved. To avoid isotopic discrimination during cutting, as reported by Juchelka et al. (23), the cut is chosen to be rather broad and both ionones are transferred by the same cut.

In Figures 9 and 10 the $\delta^{13}\text{C}_{\text{V-PDB}}$ values are plotted versus the $\delta^2\text{H}_{\text{V-SMOW}}$ values of (*E*)- α -ionone and (*E*)- β -ionone, respectively. All investigated synthetic samples do not fit the authenticity ranges of raspberry samples. The (*E*)- α -ionone declared to be "natural" shows isotopic values of both $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$ that correspond with the literature data of (*E*)- α -ionone from biotechnology. However, this "natural" (*E*)- α -ionone sample was detected to be a racemate. This fact is in fundamental contradiction to enzyme-catalyzed biosynthesis, which generally has been reported to be rather enantioselective favoring either (*R*)- or (*S*)-configuration. In the case of (*E*)- α -ionone exclusively the (*R*)-enantiomer (>>99%) is reported in the literature as the natural flavor compound. The isotopic values of the nonchiral (*E*)- β -ionone standard (declaration "natural") are comparable with the investigated "natural" (*E*)- α -ionone. Even if a final decision on the legal status of these compounds cannot be given, the correlations of $\delta^{13}\text{C}/\delta^2\text{H}$ values clearly prove that (*E*)- α (β)-ionone, declared to be "natural", does not originate from raspberries (Figures 9 and 10).

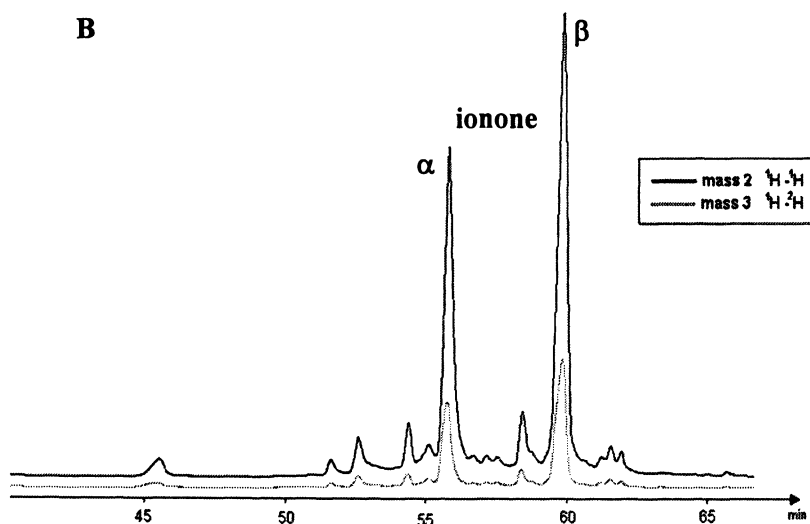
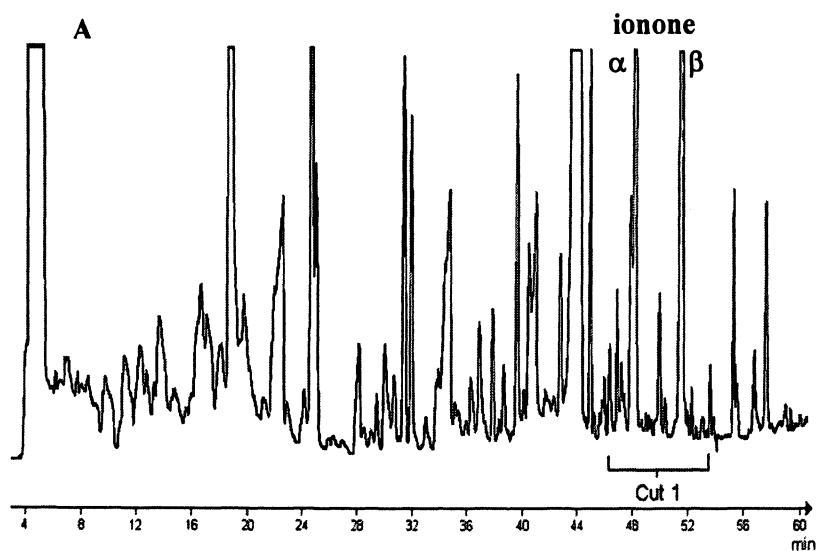


Figure 8. Precolumn (A, FID) and main column (B, SIM detection) chromatogram of a raspberry extract. (Reproduced from reference 49. Copyright 2005 American Chemical Society.)

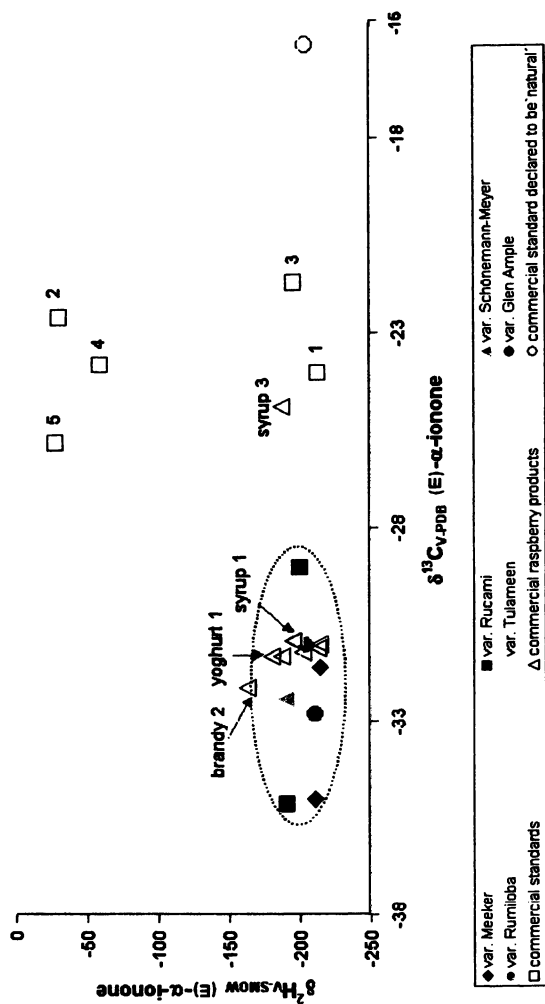


Figure 9. Correlation of $\delta^2 H_{VSMOW} / \delta^{13} C_{V-PDB}$ values of (E)- α -ionone (‰). (Reproduced from reference 49. Copyright 2005 American Chemical Society.)

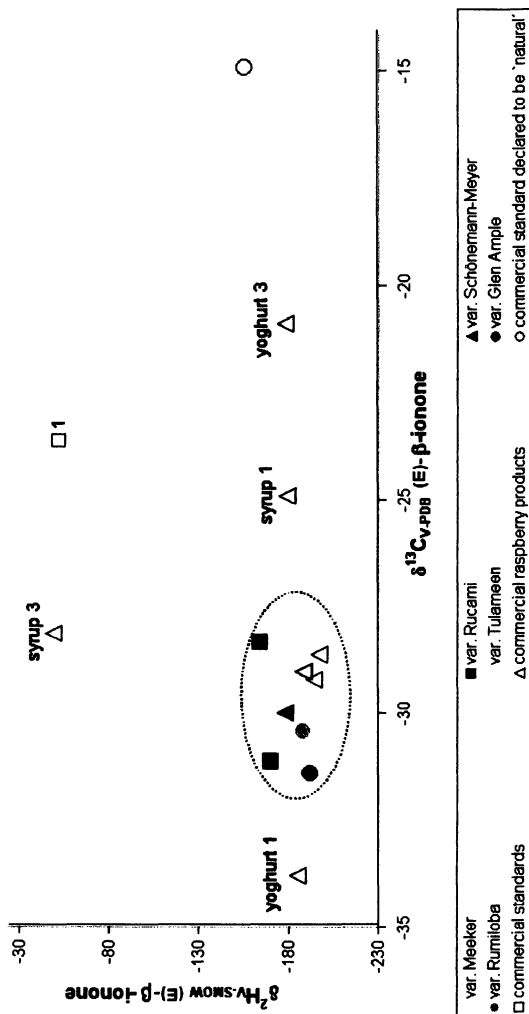


Figure 10. Correlation of $\delta^2 H_{v,SMOW} / \delta^{13} C_{v,PDB}$ values of (E)- β -ionone (‰). (Reproduced from reference 49. Copyright 2005 American Chemical Society.)

Most of the investigated raspberry products fit well with the authenticity range of the raspberries (Figures 9 and 10); however, some products show deviating isotopic values.

Concluding remarks

The combination of $\delta^{18}\text{O}_{\text{V-SMOW}}$ with $\delta^2\text{H}_{\text{V-SMOW}}$ and $\delta^{13}\text{C}_{\text{V-PDB}}$ values as a multielement analysis provides more explicit information, and authenticity assessment of natural compounds will become more reliable and conclusive.

In summary, the presented data show that the constant flow technique MDGC-C/P-IRMS provides accurate and precise $\delta^2\text{H}_{\text{V-SMOW}}$, $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^{18}\text{O}_{\text{V-SMOW}}$ values. These investigations highlight that the performance of multielement/multicomponent IRMS techniques in conjunction with enantio-MDGC/MS measurements are mandatory for comprehensive authenticity assessment in flavor analysis.

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Chapter 5

Flavor Authenticity Studies by Isotope Ratio Mass Spectrometry: Perspectives and Limits

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In recent years authenticity assessment of flavor substances has gained increased importance by using multi-element isotope ratio mass spectrometry (IRMS). Whereas $^{13}\text{C}/^{12}\text{C}$ ratio determinations have already been performed previously by coupled gas chromatography (GC) in the combustion (C) mode, the technical prerequisites for GC-pyrolysis (P)-IRMS were made available only recently. Thus, the additional information about the $^2\text{H}/^1\text{H}$ and $^{18}\text{O}/^{16}\text{O}$ ratios of industrially attractive flavor compounds opened the way to multi-element approaches. From the current studies conclusions can be drawn concerning (i) general and (ii) element-specific requirements for GC-C/P-IRMS measurements. They comprise for (i) the availability of authentic reference material, statistically relevant sample numbers, the exclusion of isotope discrimination in the course of sample preparation and chromatographic steps, as well as continuous checks of system stability using certified standards. As to (ii), GC-C-IRMS measurements of $^{13}\text{C}/^{12}\text{C}$ ratios are routinely performed; the GC-P-IRMS determination of $^2\text{H}/^1\text{H}$ ratios is a highly promising technique provided that the dynamic linearity is checked carefully and, depending on the structure of the target molecule potential isotope exchange is excluded. GC-P-IRMS measurements of $^{18}\text{O}/^{16}\text{O}$ ratios still suffer from the empirical pyrolysis technique and the need to use tertiary standardization. In addition, similarly to $^2\text{H}/^1\text{H}$ determinations, check of dynamic linearity and potential isotope exchange is required. Considering these requirements, GC-P-IRMS is an additional helpful tool in the authenticity assessment, in particular, as predictions about the global $\delta^{18}\text{O}_{\text{VSMOW}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ values of natural compounds on the basis of their biogenesis are increasingly available.

The problem of authentication

The quality of food is mainly determined by its aroma which is an important part of the flavor, *i.e.* the complex sensory impression of odor, taste, chemosensation and texture that appears during eating. Constituents of flavors are the volatile, chemically exactly defined flavor compounds. Approximately 8000 volatile substances have been found in food flavors to date (1); about 2000 industrially used materials will be integrated in a list according to E1999/217/EWG by the EU (2). Once this 'positive list' is in effect, the EU will have taken a similar approach regarding the approval of flavor materials in foods and beverages, as the USA where the so-called FEMA-GRAS list (a positive list with a little more than 2000 flavoring materials) has been in force for many years.

All constituents of industrially produced flavors are governed by legislation and codes. In the 1998 directive, the EU defined six classes of flavoring substances, *i.e.* (i) natural compounds; (ii) nature-identical compounds; (iii) artificial compounds; (iv) flavor extracts; (v) processed flavors; and (vi) smoked flavors (3). If we focus our interest on the first two groups, we will define: *Natural flavor compounds* are substances that are obtained only from suitable natural raw materials of plant or animal origin by means of appropriate physical processes, (including distillation and solvent extraction), or through microbiological or enzymatic, *i.e.* biotechnological processes. *Nature-identical flavor compounds* are substances that are obtained *via* chemical synthesis or through isolation from natural products by means of chemical processes. The chemical structure of nature-identical flavor compounds is identical to that of the corresponding natural ones. In order to be considered nature-identical, the flavor compound has to have been identified in plant or animal material traditionally consumed by humans as food. In addition to the EU Directive the IOFI Guidelines are worth mentioning in which an interpretation in more detail is given about the conditions and processes used by the flavor industry on a global basis.

Flavor compounds from these two categories differ in their market values. Between nature-identical substances and natural ones average price differences ranging from 1:10 to 1:100 exist. As a consequence, there is a certain temptation to increase the profit by unlawfully giving false declarations, *e.g.* declaring a nature-identical flavor compounds as natural. Both the industry and the consumer are confronted with this situation, the first when buying raw materials to be used in the composition of a food flavor, the latter when selecting the flavored product at the supermarket.

How can the question be answered whether the content corresponds to the declaration? Principally, differences of selected parameters arising between nature and laboratory chemistry are analytically evaluated. Firstly, the well-known selectivity of nature is used to biosynthesize preferably one of the

enantiomers of a chiral compound, while chemical synthesis – except for asymmetric modifications – leads to an enantiomeric ratio of 50:50. Gas chromatographic (GC) techniques are established to exactly determine the enantiomeric ratio of flavor compounds (4), but they are limited to the small amount of chiral flavor substances. Secondly, the determination of the ratio of stable isotopes is the method of choice to obtain more comprehensive information. Once the analytical information has been collected, it needs to be carefully interpreted, and the decision needs to be made whether the respective natural material has been indeed made from natural starting materials in a process considered natural.

The exact determination of the extremely small differences in isotope abundances can be realized by a precise quantitative mass spectrometric method, *i.e.* the isotope mass ratio spectrometry (IRMS) applicable to simple gases (5). They have to be produced from the organic compounds by combustion (C) or/and pyrolysis (P). The measuring gases needed are CO₂ formed in the C-mode as well as hydrogen gas and CO, the latter two generated in the P-mode.

The prerequisite for IRMS measurements of complex mixtures of flavor compounds in food is their separation and purification. Separation into pure compounds and IRMS analysis can be performed by online combination of GC with IRMS. The integrated interface consists of two different reactor types, one to realize the combustion of carbon into CO₂ (for ¹³C/¹²C analysis) and two others to pyrolyze the compounds, forming H₂ and CO as measuring gases for ²H/¹H and ¹⁸O/¹⁶O determinations, respectively (Fig. 1). The isotope ratios are expressed in per mil (‰) deviation relative to the Vienna Pee Dee Belemnite (VPDB) and Vienna Standard Mean Ocean Water (VSMOW) international standards.

Mass spectrometrical measurements of isotope ratios were limited to “off-line” determinations of ¹³C/¹²C and ²H/¹H ratios for a long time. In the past decade, the “on-line” coupling of GC with IRMS via a combustion interface has opened the access to the ¹³C/¹²C ratios of individual constituents in complex flavorings (5). Recently, the measurement of ¹⁸O/¹⁶O ratios was made available in both “off-line” and “on-line” modes using P-IRMS (6-10).

The large variations known to exist in the ²H/¹H ratio in nature have made it a very attractive target for IRMS studies. However, technical problems have precluded for a long time successful measurement of ²H/¹H ratios of individual peaks eluting from a capillary column. Recently, these problems have been overcome, and ²H/¹H determinations of GC peaks are possible using commercially available equipment (11,12). At present, multi-element isotope information is available about a number of ‘key’ flavor substances, *e.g.*, citral (13), (*E*)-2-hexenal, (*E*)-2-hexenol, and decanal (14), linalool and linalyl acetate (15,16), as well as several aromatic volatiles like estragol and methyl eugenol (17). In the following, several selected examples of GC-IRMS analyses are represented and the recent progress of coupled IRMS modes is highlighted.

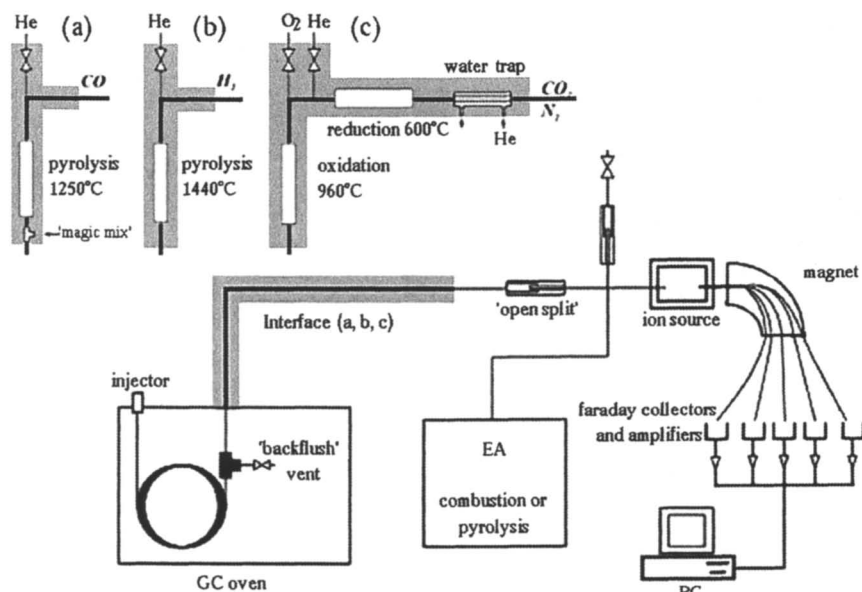


Figure 1. GC-IRMS-System (scheme). The system consists of a gas chromatograph coupled to an isotope ratio mass spectrometer via a pyrolysis or combustion interface (a, b and c for oxygen, hydrogen, and carbon measurements, respectively) and is additionally equipped with an elemental analyzer (EA).

GC-IRMS measurements: From single to multi-element approach

If we focus first on the determination of $^{13}\text{C}/^{12}\text{C}$ ratio we will notice that this technique has a traditional value in the differentiation of flavor compounds arising from C3 and C4 plants (18) (or CAM – performing the so called crassulacean acid metabolism, such as vanillin (19)). For instance, the $\delta^{13}\text{C}_{\text{VPDB}}$ values of 2-methylpropanol formed by alcoholic fermentation of sucrose from C3 and C4 sources show the expected (5) ranges of -24.7 to -21.4‰ and -12.4 to -9.2‰ for the product from C3 and C4 origin, respectively. By adding step-by-step cane sugar (C4) to the beet sugar (C3) before fermentation, an increase in the $\delta^{13}\text{C}_{\text{VPDB}}$ values of 2-methylpropanol results (Fig. 2) (20).

Literature reveals that authenticity assessment performed solely by means of $^{13}\text{C}/^{12}\text{C}$ ratio is in most cases not successful within the area of flavorings from C3 origin, so the above-mentioned introduction of technical prerequisites for GC coupled IRMS of $^2\text{H}/^1\text{H}$ ratio (11,12) was a milestone in flavor authenticity

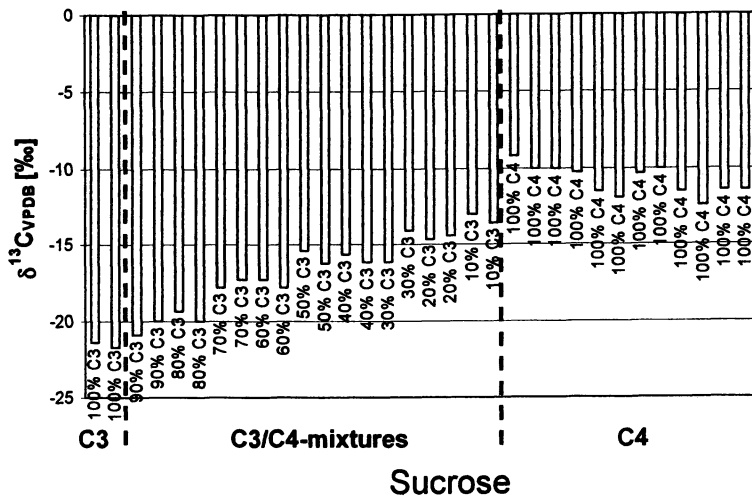


Figure 2. $\delta^{13}\text{C}_{\text{VPDB}}$ values [‰] of 2-methylpropanol recorded by GC-C-IRMS after fermentation of beet sugar (C3) and cane sugar (C4). The amount of C3/C4 source in every sample is indicated. Standard deviation: $\pm 0.1\%$ (Reproduced from reference 19. Copyright 1997 American Chemical Society.)

studies. The high potential of the technique was first demonstrated by means of benzaldehyde arising from various sources (21). As expected from results obtained by 'off-line' techniques (5,22,23), differentiation between natural and synthetic origin was realized, for the first time by direct IRMS analysis without time-consuming and laborious separation and purification of the pure target compound. As shown in Figure 3, the $\delta^2\text{H}_{\text{VSMOW}}$ values obtained by GC-P-IRMS from plant material such as fruits, kernels, leaves as well as bitter almond oils (range: -189 to -111‰) fitted with those of natural references (-164 to -83 ‰). The group of commercially available products under study, e.g., processed cherries, dairy products and various beverages, showed an inhomogeneous distribution of their $^2\text{H}/^1\text{H}$ ratios indicating, in part, several adulterations.

Similar results were obtained in our GC-IRMS authenticity studies of another industrially attractive flavor compound, (*E*)-methyl cinnamate (24). Fundamental information obtained by $^{13}\text{C}/^{12}\text{C}$ and $^2\text{H}/^1\text{H}$ measurements is provided in Figs. 4 and 5. As shown from the correlation of $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ values in Fig. 4, clearcut differentiation between synthetic (*E*)-methyl cinnamate and that of different natural sources was obtained.

In order to check the influence of the building blocks of the ester, methanol and cinnamic acid, also the IRMS data of these (commercially available) compounds were investigated. The results obtained are represented in Fig. 5. For

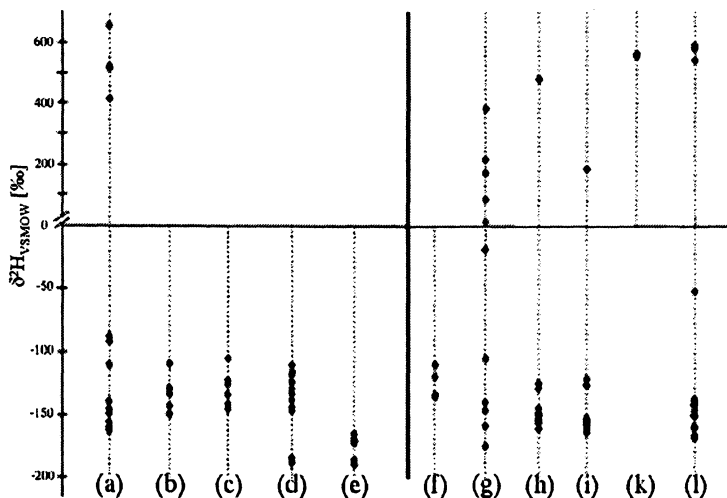


Figure 3. $\delta^2\text{H}_{\text{VSMOW}}$ values [‰] of benzaldehyde recorded by GC-P-IRMS originating from (a) synthetic and 'natural' references, (b) bitter almond oils, (c) fruit pulps, (d) fruit kernels (e) leaves, (f) processed cherries, (g) dairy products, (h) nectars, (I) other beverages, (k) aromatized teas as well as (l) liqueurs. Standard deviation: $\pm 5\text{‰}$ (20).

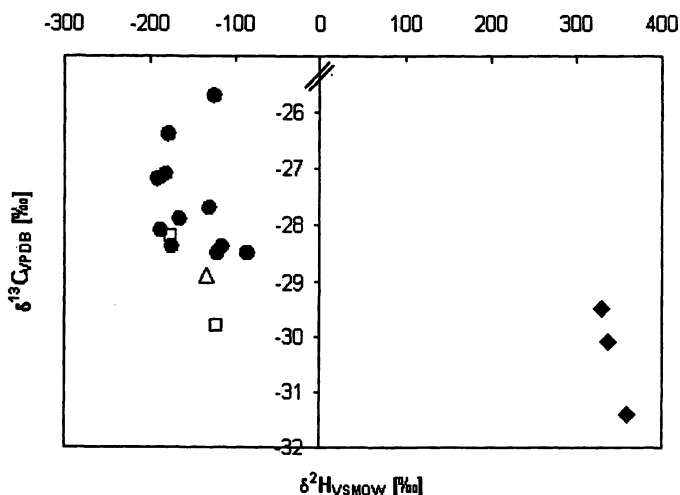


Figure 4. Correlation of $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ values [‰] of (*E*)-methyl cinnamate from synthetic reference (\blacklozenge), natural reference (\triangle), basil extract (\square), and commercial aromas declared to be natural (\bullet). Standard deviations: $\pm 0.1\text{‰}$ and $\pm 5\text{‰}$ for $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ determinations, respectively (Reproduced from reference 23. Copyright 1997 American Chemical Society.)

synthetic methanol $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ values ranged from -30.8 to -42.0‰ and from -18 to -138‰, respectively. For natural methanol, data ranging from -29.2 to -30.2‰ and from -188 to -227‰ for $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^2\text{H}_{\text{VSMOW}}$, respectively, were found. Differences were also observed between synthetic ($\delta^{13}\text{C}_{\text{VPDB}}$ from -29.1 to -30.8‰ and $\delta^2\text{H}_{\text{VSMOW}}$ from +421 to +472‰) and natural cinnamic acid ($\delta^{13}\text{C}_{\text{VPDB}}$ from -25.6 to -26.2‰ and $\delta^2\text{H}_{\text{VSMOW}}$ from -124 to -156‰). As synthetic (*E*)-methyl cinnamate is known to be produced by oxidation of benzaldehyde (produced from toluene), these data are in good agreement with that of synthetic benzaldehyde (21).

Another example of successful application of recent multi-element GC-IRMS authentication (25) is pear flavor that is dominated by 2,4-decadienoates as ‘impact’ compounds. Correlations of $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ data of methyl *E,Z*-2,4-decadienoate and ethyl *E,Z*-2,4-decadienoates (each originating from various sources) are represented in Figs. 6 and 7, respectively. As shown from both figures, the GC-C/P-IRMS techniques provided distinct authentication of the pear esters originating from synthetic to ‘ex fruit’ sources. Interestingly, the isotope data of the esters from brandy were found to be slightly enriched. Such inverse isotope effects have already been observed many years ago in the course of liquid-vapour transformation of alcohols (26).

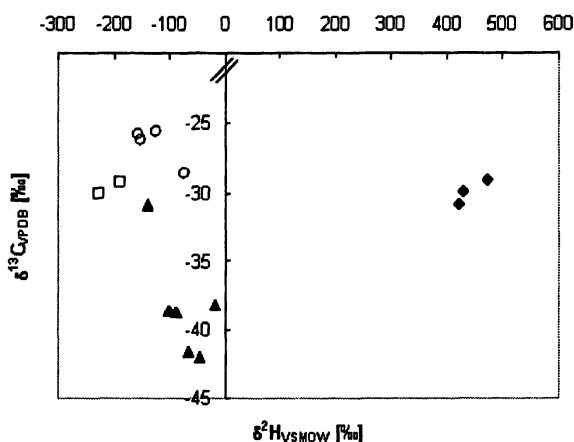


Figure 5. Correlation of $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ values [‰] of synthetic methanol from gas (\blacktriangle), natural methanol (\square), natural cinnamic acid (\circ), and synthetic cinnamic acid (\blacklozenge). Standard deviations: $\pm 0.1\text{‰}$ and $\pm 5\text{‰}$ for $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ determinations, respectively. (Reproduced from reference 23. Copyright 1997 American Chemical Society.)

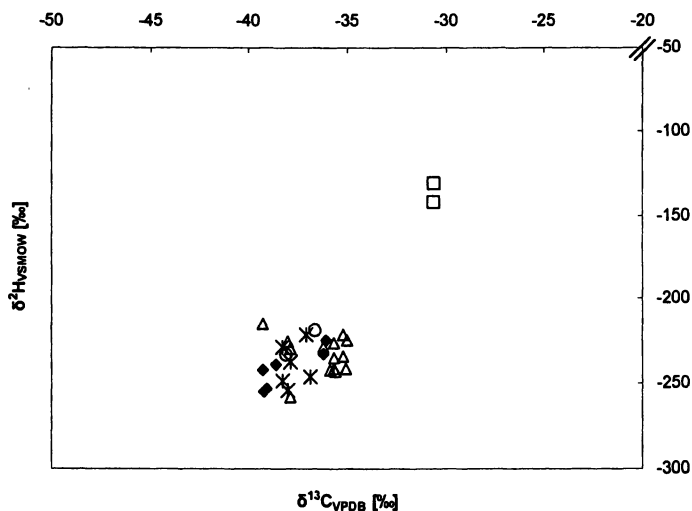


Figure 6. Correlation of $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ values [‰] of methyl *E,Z*-2,4-decadienoate from pear fruit (\blacklozenge), pear juice (\circ), baby food ($*$), brandy (Δ) as well as synthetic references (\square). Standard deviations: ± 0.1 and $\pm 5\text{‰}$ for $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ determinations, respectively (Reproduced from reference 24. Copyright 2004 American Chemical Society.)

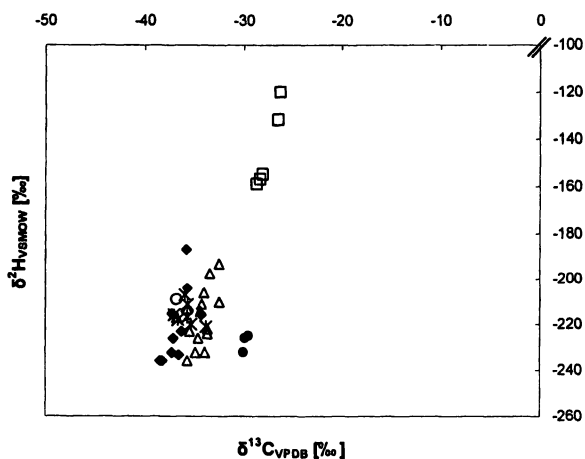


Figure 7. Correlation of $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ values [‰] of ethyl *E,Z*-2,4-decadienoate from pear fruit (\blacklozenge), pear juice (\circ), baby food ($*$), brandy (Δ) as well as natural references (\bullet) and synthetic references (\square). Standard deviations: ± 0.1 and $\pm 5\text{‰}$ for $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ determinations, respectively (Reproduced from reference 24. Copyright 2004 American Chemical Society.)

As to the results represented in Figs. 4-7, in each case the $^2\text{H}/^1\text{H}$ ratio influenced essentially the authentication. However, in order to show an example where this was not the case but, in contrast, the $^{13}\text{C}/^{12}\text{C}$ ratio was the parameter decisive for authenticity assessment, let us focus our attention on γ -decalactone (Figure 8) (27). Studying γ -decalactone from peach, apricot and nectarine, similar ranges of isotope values were observed by GC-C/P-IRMS. For these *Prunus* fruits, the $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ data ranged from -34.6 to -38.4‰ and -160 to -206‰. Synthetic references, however, showed $\delta^{13}\text{C}_{\text{VPDB}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ values ranging from -27.4 to -28.3‰ and -151 to -184‰, respectively. As can be seen additionally from Fig. 8, is the possibility of analytical discrimination of the 'ex *Prunus* plant' and biotechnological origins. Thus, decisive influence was given by the $\delta^{13}\text{C}_{\text{VPDB}}$ data.

In several cases, a correlation of $\delta^2\text{H}_{\text{VSMOW}}$ and $\delta^{18}\text{O}_{\text{VSMOW}}$ values can provide helpful authenticity assessment. As a representative example, in Figure 9 the $^2\text{H}/^1\text{H}$ and $^{16}\text{O}/^{18}\text{O}$ ratios recorded for Z-3-hexenol from various sources are represented. On the basis of the $\delta^2\text{H}_{\text{VSMOW}}$ values alone, no distinction between the origins of the samples can be made. Only by including the $\delta^{18}\text{O}_{\text{VSMOW}}$ values a definite authentication can be performed between the natural samples compared to the nature-identical references.

Conclusions

From the information provided by the literature and the data highlighted in this overview, conclusions can be drawn concerning (i) general and (ii) element-specific requirements for GC-C/P-IRMS measurements. They comprise for (i) the availability of authentic reference material, statistically relevant sample numbers, the exclusion of isotope discrimination in the course of sample preparation and chromatographic steps, as well as continuous checks of system stability using certified standards. As to (ii), GC-C-IRMS measurements of $^{13}\text{C}/^{12}\text{C}$ ratios are routinely performed; the GC-P-IRMS determination of $^2\text{H}/^1\text{H}$ ratios is a highly promising technique provided that the dynamic linearity is checked carefully and, depending on the structure of the target molecule potential isotope exchange is excluded. GC-P-IRMS measurements of $^{18}\text{O}/^{16}\text{O}$ ratios still suffer from the empirical pyrolysis technique and the need to use tertiary standardization. In addition, similarly to $^2\text{H}/^1\text{H}$ determinations, check of dynamic linearity and potential isotope exchange is required. Considering these requirements, GC-P-IRMS is an additional helpful tool in the authenticity assessment, in particular, as predictions about the global $\delta^{18}\text{O}_{\text{VSMOW}}$ and $\delta^2\text{H}_{\text{VSMOW}}$ values of natural compounds on the basis of their biogenesis are increasingly available (28,29).

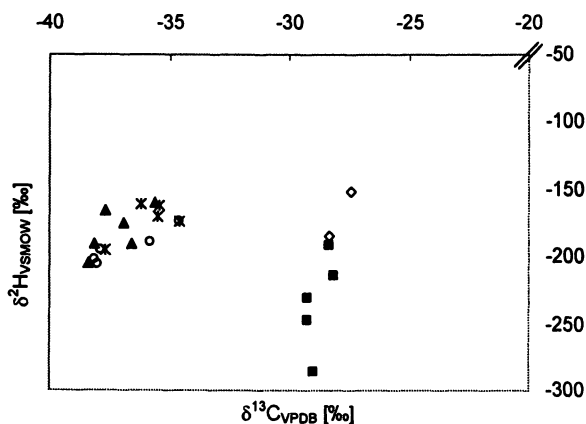


Figure 8. Correlation of $\delta^{13}C_{VPDB}$ and δ^2H_{VSMOW} values [‰] of γ -decalactone from peach (\blacktriangle), nectarine ($*$), and apricot (\square) fruits, and synthetic (\blacksquare) as well as natural (\blacklozenge) references. Standard deviations: ± 0.2 - 0.3 ‰ and ± 5 ‰ for $\delta^{13}C_{VPDB}$ and δ^2H_{VSMOW} determinations, respectively (Reproduced with permission from reference 26. Copyright 1990.)

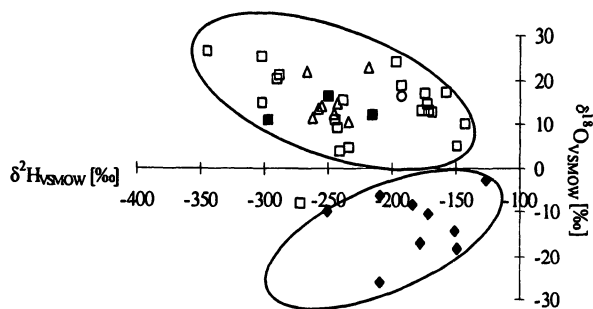


Figure 9. Correlation of $\delta^{18}O_{VSMOW}$ and δ^2H_{VSMOW} values [‰] of Z-3-hexenol from vegetables (\blacksquare), leaves/blossoms (Δ), cactus pear fruits (\circ), and natural (\square) as well as 'nature-identical' (\blacklozenge) references. Standard deviations: ± 0.5 ‰ and ± 5 ‰ for $\delta^{18}O_{VSMOW}$ and δ^2H_{VSMOW} determinations, respectively.

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Chapter 6

Authentication of Essential Oils

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Essential oils are commonly defined as complex mixtures of flavor and fragrance substances originating from plants. The authenticity of natural compounds is an important topic for the flavor and fragrance industry because of legal or commercial aspects. Over the last 15 years stable isotope analysis has become the most important tool for authenticity testing of non-chiral compounds. The analysis of enantiomeric purity is well-known as an efficient possibility for the authenticity control of chiral compounds. It is of great interest to check whether it is possible to detect the blending with synthetic substances or with isolates from other essential oils. In this paper we present examples for the use of both methods for authentication of laurel leaf (α -terpinyl acetate), oregano (carvacrol), ylang ylang (*p*-cresyl methyl ether, benzyl acetate) and sandalwood oil (*cis*- α -santalol). This publication demonstrates the merits of enantioselective capillary GC and isotope ratio mass spectrometry (IRMS), coupled on-line with capillary GC for the authentication process.

Essential oils are valuable flavor and fragrance raw materials provided by an enormous variety of plants from different geographical regions. Seasonal changes lead to the need for standardization, which can be achieved by means of distillation and subsequent fractionation, pooling of qualities as well as the addition of synthetic key compounds. In order to avoid fraud in the trade of commercial qualities, various techniques have been developed for the authenticity control of essential oils. Besides GC/MS analysis, chiral separation of enantiomers and isotope ratio mass spectrometry (IRMS) are enjoying increasing attention (1-4). In this study 2 essential oils, which are widely used for flavor application, oregano oil and laurel oil, as well as 2 essential oils which are important constituents of many fine fragrance products, sandalwood oil and ylang-ylang oil, were studied.

Experimental Section

Essential oil samples

The total of 13 oregano oil samples originate mainly from *Origanum vulgare* L. *ssp. hirtum*, the so-called Greek oregano, from *Origanum onites* L., the Turkish oregano, and from *Coridothymus capitatus* synonymous with *Thymbra capitata* (L.) Cav, or *Thymus capitatus*, the so-called Spanish oregano. All species belong to the Lamiaceae family and grow mainly in the Mediterranean area. Oregano leaves from Turkey [harvest 20031], 3 different samples of cut oregano from experimental fields (Bundesanstalt für Züchtungsforschung, Quedlinburg, Germany), 5 of cut oregano from experimental fields in Artern (Germany) and 2 samples (commercial crops) from cooperative farmland in Eisleben (Germany) were used for steam distillation via SDE (Simultaneous Distillation Extraction after Likens-Nickerson). Two commercial oregano oils were received from Albert Vieille S.A. (Vallauris, France) and Adrian Industries (harvest 2003, Aix en Provence, France).

Commercial samples of carvacrol, 5-isopropyl-2-methyl phenol [CAS 499-75-2] were purchased from Munoz Galvez (Murcia, Spain). Additional qualities were synthesized starting from L-carvone.

Two commercial laurel leaf oils originating from the Dalmatian coast (former Yugoslavia) and two oils obtained by SDE in our laboratory from leaves from Turkey (Unterweger, Austria) and France (Payan Bertrand, France) respectively were analyzed.

α -Terpinyl acetate [CAS 80-26-2] was purchased from DRT (Dax, France) and Millennium (Jacksonville, USA).

Some 12 Sandalwood oils were checked for this survey: 7 commercial qualities of *Santalum album* (suppliers: Charabot, Grasse, France; India Sales, Chennai, India; Frey & Lau, Henstedt-Ulzburg, Germany; Polarome, Basel, Switzerland), 3 commercial qualities of *S. austrocaledonicum* (suppliers: A. Vieille; Kaders, Hamburg, Germany; Erbslöh, Velbert, Germany), and 1 of *S. spicatum* (supplier: Australian Sandalwood oil company, Quai de Versailles, France), 1 laboratory quality of *Santalum album* var. *marchionense*.

For the comparison of ylang-ylang and cananga oils 4 commercial qualities of ylang-ylang oils of different grades and 1 cananga oil sample were analyzed (suppliers: Frey & Lau, Adrian, Kaders), *p*-cresyl methyl ether (Symrise), and benzyl acetate (Tessenderlo, Zurzach, Switzerland).

All essential oil samples and the aroma chemicals were analyzed using GC/MS, GC/C-IRMS and GC/TC-IRMS.

Instrumental analysis

Instrumentation (capillary gas chromatography, spectroscopy) as well as analytical and preparative conditions have been described in a previous publication (5). For chiral separations, a fused silica column (25 m x 0.25 mm, film thickness 0.25 μm) from MEGA capillary columns laboratory (Legnano, Italy) was used. The commercially available column was coated with a solution of di-methyl-pentyl-gamma-cyclodextrin 30% in OV 1701. As a second column for the separation of α -terpinyl acetate an octakis-(2,3-di-*O*-pentyl-6-*O*-methyl)-gamma-cyclodextrin column (Macherey & Nagel, Düren, Germany) was successfully tested.

Isotope Ratio Mass Spectrometry

The on-line analysis of $\delta^{13}\text{C}_{\text{V-PDB}}$ values in the effluent from a GC column is achieved by the conversion of organic molecules to CO_2 , which is subsequently analyzed by a dedicated mass spectrometer (GC combustion IRMS, GC/C-IRMS). For oxygen-18 measurements CO as a conversion product is preferred. At a temperature of 1280 $^\circ\text{C}$ organic molecules are converted to CO , H_2 and C by a specific thermo chemical conversion reactor. Applying this process GC/TC-IRMS (Gas Chromatography / High Temperature Conversion) enables the calculation of the $^{18}\text{O}/^{16}\text{O}$ ratio in the molecule and $\delta^{13}\text{C}_{\text{V-PDB}}$ data, as well. For deuterium/hydrogen measurements a conversion reactor was employed at a temperature of 1450 $^\circ\text{C}$.

Results and Discussion

Oregano Oils

Oregano oils are produced by steam distillation from leaves and blossoms of 60 species of the Lamiaceae family, growing mainly in the Mediterranean area. The oils originate mainly from *Origanum vulgare* L. ssp. *hirtum*, the so-called Greek oregano, from *Origanum onites* L., the Turkish oregano, and from *Coridothymus capitatus* synonymous with *Thymbra capitata* (L.) Cav. or *Thymus capitatus*, the so-called Spanish oregano (6). Additional commercial qualities are produced in North America, Asia and South America. The herb material itself is used as a seasoning for pizza, for meat and fish dishes. The essential oil (yield up to 3%) is characterized by a strong, phenolic, spicy, herbal, leathery, bitter smell and taste, which is mainly represented by carvacrol. Nowadays in addition to steam- or hydrodistillation also sub- and supercritical CO₂-extraction is used to produce oregano oils. Supercritical fluid extraction is used for fractionation and deteipenation of the oil (7). For oregano oils antimicrobial, antioxidant, antifungal, cytotoxic, insecticidal, and nematicidal activities are reported. In many cases carvacrol (1) is accompanied by thymol (2) in these oils. Recently, a mixture of both was claimed to possess a synergistic, bactericidal effect against *Tropnema*, which can induce diseases of cattle (8). This indicates strong interest of the pharmaceutical industry in oregano oils. In 2004 prices of oregano oils ranged from about 100 to 280 US-\$/kg and from 30 to 80 US-\$/kg for synthetic carvacrol. Carvacrol can be synthesized starting from carvone. When treated with strong acids, carvone isomerizes to carvacrol (9).

Lawrence reports that oils from different botanical species contain carvacrol in the range from 40 to over 80% (10). For Mexican oregano produced from *Lippia graveolens* HBK (Verbenaceae) he published carvacrol contents from 3 to over 20% (11). Commercial database records a carvacrol range from 0.6 to 86.3% (12). This large range can be found since e.g., *Thymus capitatus* oil is produced from plants collected in their wild state, which is represented by 3 chemotypes, one carvacrol-rich, one thymol-rich and one mixed type (13).

An ISO final draft for an International Standard with regard to the Spanish type of oregano oil originating from *Thymbra capitata* (L.) Cav. exists. ISO gives a type chromatogram and the identification for 11 peaks. Carvacrol is shown as main ingredient without concentration limits (14). E.O.A. also specifies the Spanish type and gives limits for the content of phenolic compounds from 60 to 75% (15).

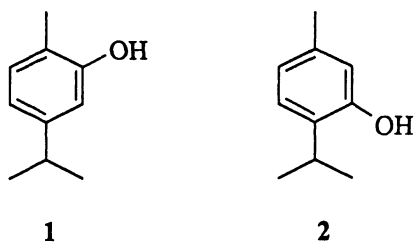


Figure 1: Chemical structures of carvacrol (1) and thymol (2).

Since commercial qualities of carvacrol are relatively pure, the analysis of byproducts does not provide sufficient data. The check for markers performed with a N- and S-selective detector led to the detection of trace amounts of dimethyl sulfone in 2 of 3 analyzed oils. This finding, however, does not provide enough evidence for authenticity control. In the past on-line gas chromatography thermo chemical conversion isotope ratio mass spectrometry (GC/TC-IRMS) was applied for the differentiation of authentic and synthetic phenolic compounds in oils and extracts. Fink et al. analyzed (*E*)-methyl cinnamate in basil extract (16). Ruff et al. conducted a broad study of estragole and eugenol methyl ether in tarragon oil, sweet basil oil, pimento oil, and laurel leaf oil (17). Bilke et al. applied this technique for the analysis of *trans*-anethole in anise and fennel oils (18). In all publications the characterization of organic molecules was based on the determination of the stable isotopes of the bioelements C, H, O, since the one dimensional dataset e.g. for carbon-13, does not provide enough information for the differentiation of molecules coming from the same plant category (cf. Table I).

Table I. Plant Categories Regarding CO₂-Fixation during Photosynthesis (19).

CO ₂ fixation	Example	$\delta^{13}\text{C}_{\text{CV-PDB}}$ [‰]
C ₃ plants	wheat, barley, sugar beet, rice	-24...-32
CAM plants	succulents, orchids, pineapple	-12...-30
C ₄ plants	sugar cane, corn, sorghum	-10...-16

IRMS data are usually expressed relative to an international standard, which is represented by the fossil carbonate PDB (V-PDB, Vienna Pee-Dee Belemnite) for the ratio of carbon-13 to carbon-12. All values are expressed in the δ -notation:

$$\delta^{13}\text{C}_{\text{V-PDB}}[\text{‰}] = \left(\frac{[^{13}\text{C}_{\text{sample}}]/[^{12}\text{C}_{\text{sample}}]}{[^{13}\text{C}_{\text{standard}}]/[^{12}\text{C}_{\text{standard}}]} - 1 \right) \cdot 1000$$

The combination with $\delta^{18}\text{O}$ data provides a second dimension and the introduction of $\delta^2\text{H}$ data a third dimension. For ^{18}O and ^{21}H measurements V-SMOW (Vienna Standard Mean Ocean Water) is the approved international standard.

Table II illustrates that the range of $\delta^{13}\text{C}_{\text{V-PDB}}$ data for carvacrol from synthetic source with -31.0‰ to -29.9‰ and from oregano oils with -31.0‰ to -27.7‰ is completely overlapping, while the corresponding $\delta^2\text{H}_{\text{V-SMOW}}$ data for synthetic material with -328‰ to -285‰ and natural origin with -381‰ to -337‰ show no overlapping with regard to a total dispersion of 96‰. The $\delta^{18}\text{O}_{\text{V-SMOW}}$ data range for synthetic $+7.2\text{‰}$ to $+9.3\text{‰}$ and natural qualities with $+10.0\text{‰}$ to $+13.1\text{‰}$ show no overlap. The $\delta^2\text{H}_{\text{V-SMOW}}$ dataset of synthetic qualities A and B separates significantly from the corresponding $\delta^2\text{H}_{\text{V-SMOW}}$ data of natural oils. In contrast the synthetic quality C, derived from synthetic carvone, can be well differentiated from the natural oils by its $\delta^{18}\text{O}_{\text{V-SMOW}}$ data.

In parallel an investigation of thymol in oregano oil samples was conducted. It was found that the $\delta^2\text{H}_{\text{V-SMOW}}$ data for authentic material with -428‰ to -300‰ is significantly lower than the 1 synthetic sample with -124‰ .

Laurel Leaf Oil

Laurus nobilis L., an evergreen tree or shrub, belongs to the family of Lauraceae. It grows in Asia Minor and around the Mediterranean Sea. Its dried or fresh leaves are commonly known as a household culinary herb while the essential leaf oil is mostly used for flavors and fragrances. In the food industry laurel leaves are preferred for seasoning meat products and soups. Besides avocado oil and cinnamon oil it is a commercially important representative of the Lauraceae family. The tree is cultivated primarily in Mediterranean countries and grows to a height of 5 to 10 meters. It should not be confused with the West Indian bay tree or Californian bay laurel. Laurel leaf oil has a fresh-green, strong, sweet-spicy, aromatic-eucalyptaceous odor and a fresh, delicate spicy, camphoraceous-eucalyptaceous taste. The key odorants of the leaves were characterized recently by means of aroma extract dilution analysis as (*Z*)-3-hexenal, eugenol, 1,8-cineole, and linalool (20). In the E.O.A. specification the main ingredients are covered (21). Recently, B. M. Lawrence has reviewed laurel leaf oil (22, 23) and reported large variations of the main ingredient 1,8-cineole from 25 to 70%, followed by α -terpinyl acetate, ranging between 5 to 15%, which might originate from seasonal variations or from different geographical origins. Since these variations are able to generate problems in

Table II: Isotopic data of natural and synthetic carvacrol.

sample	$\delta^{13}\text{C}_{\text{V-PDB}}$ [‰]	$\delta^2\text{H}_{\text{V-SMOW}}$ [‰]	$\delta^{18}\text{O}_{\text{V-SMOW}}$ [‰]
	s.d.: 0.3‰	s.d.: 10‰	s.d.: 1.0‰
ex oregano A	-28.3	-366	11.4
ex oregano B	-28.5	-366	12.4
ex oregano C	-27.9	-358	13.1
ex oregano D	-30.7	-365	11.4
ex oregano E	-31.0	-376	10.7
ex oregano F	-30.6	-381	11.6
ex oregano G	-30.2	-369	12.5
ex oregano H	-29.9	-337	11.3
ex oregano I	-27.9	-351	11.6
ex oregano J	-27.7	-347	12.3
ex oregano leaf oil comm. A (Turkey)	-28.4	-377	10.9
ex oregano oil comm. B	-28.8	-359	10.1
ex oregano oil comm. C	-29.4	-339	10.0
carvacrol synth. A	-30.3	-285	9.3
carvacrol synth. B	-31.0	-289	7.2
carvacrol synth. C	-29.9	-328	8.1

finished consumer goods, qualities are often pooled and sometimes standardized. In 2004 the price of laurel leaf oil was approx. 100 US-\$/kg, whereas 1,8-cineole ex eucalyptus or camphor oils amounted to 10 US-\$/kg and α -terpinyl acetate of synthetic origin about 4.3 US-\$/kg.

In order to establish a database with authentic oils of known geographic origin essential oil samples were prepared using leaves from France and Turkey by hydrodistillation via SDE. α -Terpinyl acetate (**3**) is a flavor compound with herbaceous, sweet spicy bergamot odor and spicy sweet taste. It occurs in cold pressed bitter orange oil (trace level up to 0.03%), bergamot oil (trace level up to 0.31%) and as well in sweet marjoram, tea and cardamom oil with 29 to 38% (24, 25). α -Terpinyl acetate was found in laurel leaf oil between 8.5 and 10.6% (Table III).

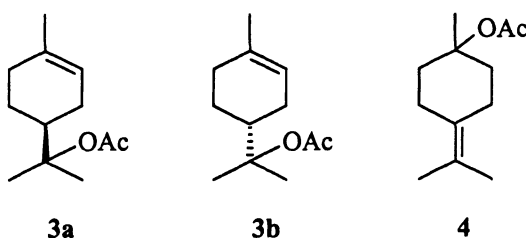


Figure 2: The chemical structures of 4S(-)- α -terpinyl acetate (**3a**), 4R(+)- α -terpinyl acetate (**3b**), γ -terpinyl acetate (**4**).

The enantioselective GC analysis of laurel leaf oil hydrodistillates from France and Turkey revealed a ratio 4S(-)- α -terpinyl acetate to 4R(+)- α -terpinyl acetate of 92.4 to 7.6 and 91.3 to 8.7, respectively. This finding is in contrast to the 80:20 ratio of two lots from a commercial oil originating from the Dalmatian coast (Table III). It is known that the enantiomers and the racemate occur in many essential oils (e.g., cardamom with about 40%, Siberian pine-needle and cypress oils) in various amounts (26). The enantioselective GC analysis revealed for synthetic qualities ratios of 50:50 and 41.4:58.6 (Table III). Commercially available synthetic terpinyl acetate can be prepared by acetylating the terpineol mixture obtained from terpin hydrate and contains other isomers such as γ -terpinyl acetate (26). Based on these findings a blend of the above mentioned seems to be possible.

The route to synthetic compounds either uses petrochemicals as starting materials or involves also products from natural sources (26). In many cases commercially competitive aroma chemicals, also called “semi-synthetic materials” are only obtained via chemical modification of natural products.

Table III. Results of the enantioselective GC analysis of α -terpinyl acetate and relative concentrations [%] of α - and γ -terpinyl acetate based on GC/MS analysis.

Samples	enantiomer 1		enantiomer 2		Conc. GC/MS [%]	
	4S(-) α -terpinyl acetate 3a [%]	4R(+) α -terpinyl acetate 3b [%]	α -terpinyl acetate 3	γ -terpinyl acetate 4		
Laurel leaf oil, commercial qual. A, Dalmatian coast (pooled)	80	20	10.65	1.22		
Laurel leaf oil, commercial qual. B, Dalmatian coast (pooled)	80	20	10.48	1.22		
Laurel leaf oil, hydrodist., (France)	92.4	7.6	10.55	-		
Laurel leaf oil, hydrodist., (Turkey)	91.3	8.7	8.51	-		
α -terpinyl acetate, synth. ex <i>Pinus pinaster</i> , (France)	50	50	57.27	22.65		
α -terpinyl acetate, synth. ex <i>Pinus spec.</i> , (USA)	41.4	58.6	80.62	15.29		

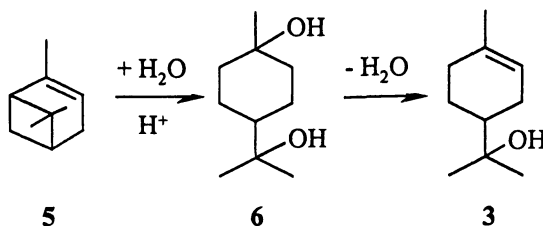


Figure 3: The synthesis of α -terpineol (3) from α -pinene (5) via terpin hydrate (6) (cf. 26).

The “semi-synthetic” character is most probably the case for the 2 commercial synthetic qualities of α -terpinyl acetate showing $\delta^{13}\text{C}_{\text{V-PDB}}$ data of -33.4‰ and -32.8‰ and $\delta^2\text{H}_{\text{V-SMOW}}$ data of -332‰ and -296‰ , respectively (Table IV). The 2 laurel leaf oil hydrodistillates from France and Turkey are characterized by slightly higher $\delta^{13}\text{C}_{\text{V-PDB}}$ data of -31.8‰ and -30.0‰ and $\delta^2\text{H}_{\text{V-SMOW}}$ data around -252‰ and -253‰ . The complete IRMS dataset of the commercial quality sample A from the Dalmatian coast is between those of natural and semisynthetic α -terpinyl acetate and supports the assumption of a blend with semisynthetic α -terpinyl acetate. Sample B shows a completely different dataset.

For further studies on laurel leaf oil linalool, eucalyptol, 1-terpinen-4-ol, eugenol methyl ether, and eugenol were analyzed via the IRMS multielement approach. Hör et al. reported for linalool ex laurel leaf oil a $\delta^2\text{H}_{\text{V-SMOW}}$ data range between -331‰ and -293‰ (27). Our $\delta^2\text{H}_{\text{V-SMOW}}$ data from authentic material were found to be -322‰ , while synthetic linalool usually is analyzed in the window between -301‰ and -207‰ (28); however, outliers like -311‰ to -156‰ and -209‰ to -159‰ were published previously (29, 30). Ruff et al. reported for eugenol methyl ether in commercial laurel oil the following dataset: $\delta^{13}\text{C}_{\text{V-PDB}}$ data -33.6‰ , $\delta^2\text{H}_{\text{V-SMOW}}$ data -107‰ and $\delta^{18}\text{O}_{\text{V-SMOW}}$ data $+2.7\text{‰}$, which show slight variation to own data for eugenol methyl ether in laurel leaf oil from France: $\delta^{13}\text{C}_{\text{V-PDB}}$ data $-33.5\pm 0.4\text{‰}$, $\delta^2\text{H}_{\text{V-SMOW}}$ data $-159\pm 15\text{‰}$ and $\delta^{18}\text{O}_{\text{V-SMOW}}$ data $+9.4\pm 1.5\text{‰}$ (17). The authors summarized that the IRMS technique did not allow differentiation of synthetic eugenol methyl ether from the product of natural origin, which was confirmed in this study. The elevated $\delta^{18}\text{O}_{\text{V-SMOW}}$ value of $+9.4\pm 1.5\text{‰}$ is in good correspondence with published data for eugenol and biogenetically related assumptions (31).

Sandalwood Oil

East Indian sandalwood oil is obtained from *Santalum album* L. and is one of the most precious raw materials for perfumery. The wood and the oil have

Table IV: GC/C-IRMS and GC/TC-IRMS data of natural and synthetic α -terpinyl acetate.

sample	$\delta^{13}\text{C}_{\text{V-PDB}}$ [‰]	$\delta^2\text{H}_{\text{V-SMOW}}$ [‰]	$\delta^{18}\text{O}_{\text{V-SMOW}}$ [‰]
	s.d.: 0.3‰	s.d.: 10-20‰	s.d.: 1.0‰
ex laurel leaf (France)	-31.8	-252	8.7
ex laurel leaf (Turkey)	-30.0	-253	11.9
ex laurel leaf oil commercial A	-32.3	-278	9.6
ex laurel leaf oil commercial B	-32.6	-240	10.2
α -terpinyl acetate synth. A	-33.4	-332	11.8
α -terpinyl acetate synth. B	-32.8	-296	10.7

been appreciated since ancient times. Both were used for religious and cultural purposes in incense, in fragrances, and in medicine. The oil possesses a powerful, woody, musky, milky-nutty, and longlasting warm scent. The annual production is estimated to be between 100 and 200 metric tons. *Santalum album* wood oil is produced mainly in India and Indonesia, but the Indian production has become unsustainable. A tree needs 25 to 40 years to grow large enough to be cut. New plantations e.g., in Australia will take about 15 years to yield harvestable wood (32). Beside the application in perfumery the use of the oil for clinical purposes and for aromatherapy is propagated (33). Due to high demand and limited availability its price increased during the last 30 years from about 40 US-\$/kg to about 800 to 1000 US-\$/kg nowadays. Despite of a broad range of synthetic sandalwood oil substitutes the demand for good quality natural oils led to the increasing importance of oils from other species like e.g. *Santalum spicatum* and *S. austrocaledonicum*. Whereas East Indian sandalwood oil is extensively studied (34), and also the oil of *S. spicatum* (35, 36, 37), less is known about the oil of *S. austrocaledonicum*. Recently we started analyses of this oil (38) in order to check the use of both species as substitutes for *S. album* oil.

The composition of the oils varies slightly from roots through the trunk to the branches. Therefore the commercial qualities show changing compositions, too. The final draft of ISO shows a type chromatogram of the oil of *Santalum album*, gives the peak identification for the 4 large sesquiterpene alcohols, and a range from 41 to 55% for *cis*- α -santalol and 16 to 24% for *cis*- β -santalol (39). In a recent publication Howes et al. suggest 43% and 18% respectively as lower limits for these alcohols (40).

The sensorially most important ingredients are *cis*- α -santalol, *cis*- β -santalol and (*Z*)- α -*trans*-bergamotol. Summarized they account for 70 to 80% in *S. album*, up to 65% in *S. austrocaledonicum* and up to 25% in *S. spicatum*. The New Caledonian oil (*S. austrocaledonicum*) contains significantly less santalols than *S. album* oil and was 100 to 250 US-\$/kg lower in price in 2004. It was the purpose of this study to check blending of commercial East Indian oils with cheaper qualities using GC/MS, enantioselective chromatography and GC/TC-IRMS. Table V shows the typical composition of commercial sandalwood oils. The relative concentration in percentage represents only average values because of large variations from batch to batch.

In order to detect a blend of e.g., 80% of *S. album* with 20% of *S. austrocaledonicum* oil, the evaluation of the gas chromatogram shows that the main peaks like *cis*- α -santalol (7) and *cis*- β -santalol (8) would still be within their limits. Further it is difficult to detect a small peak of (*E,E*)-farnesol in routine analysis, because it elutes on a polar column with *cis*- α -santalol and on an apolar column with *cis*- β -santalol. Another indicator for the above mentioned 3 oils is the ratio *cis*- α -santalol/*cis*-lanceol, which shows significant differences with values of 25, approx. 5 and 10 for *Santalum album*, *Santalum*

Table V: Typical composition of sandalwood oils: *Santalum album*, *Santalum austrocaledonicum* and *Santalum spicatum*. Relative concentrations [%] given in average values.

	<i>S. album</i>	<i>S. austrocaledonicum</i>	<i>S. spicatum</i>
<i>cis</i> - α -santalol	48	38	20
<i>cis</i> - β -santalol	20	19	9
(<i>Z</i>)- α - <i>trans</i> -bergamotol	6	9	5
α -bisabolol	0.2	0.6	5
β -bisabolol	1	1	3
<i>cis</i> -lanceol	2	9	2
<i>cis</i> -nuciferol	2	2	11
(<i>E,E</i>)-farnesol	absent	1	10
total	79.2	79.6	65

austrocaledonicum and *Santalum spicatum*, respectively. In addition the enantioselective GC analysis of β -bisabolol isomers reveals significant differences for the above-mentioned 3 oils (38).

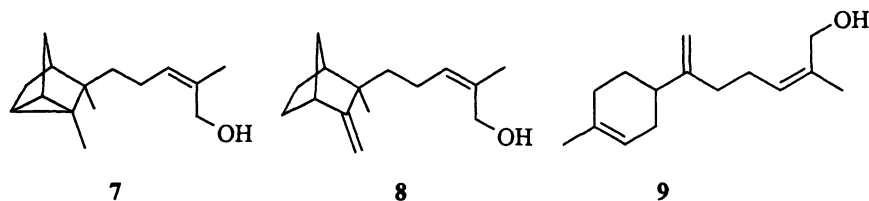


Figure 4: The chemical structures of (+)-*cis*- α -santalol (7), (-)-*cis*- β -santalol (8), (-)-*cis*-lanceol (9).

For the GC/TC-IRMS analysis of different sandalwood oils, the following compounds were investigated: (+)-*cis*- α -santalol (7), (*Z*)- α -*trans*-bergamotol, (-)-*cis*- β -santalol (8), *epi*- β -santalol, (-)-*cis*-lanceol (9) and *cis*-nuciferol. *cis*- α -Santalol (7) in the single sample of sandalwood oil Tahiti with the $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$ datapoint of -26.7‰ and -133‰ can be differentiated from the main range from -29.6‰ to -27.9‰ and -197‰ to -158‰ , respectively (Table VI). The same observation was made for (-)-*cis*- β -santalol (8). The $\delta^{13}\text{C}_{\text{V-PDB}}$ data regarding *epi*- β -santalol of East Indian sandalwood oil, sandalwood oil ex Tahiti (*Santalum album* var. *marchionense*) and *Santalum austrocaledonicum* are slightly separated with data from -32.3‰ to -29.6‰ , -28.8‰ , and -28.2‰ to -27.4‰ , respectively. For detailed interpretation, however, a broader set of samples is necessary.

Table VI: GC/C-IRMS and GC/TC-IRMS data of natural *cis*- α -santalol from different sources.

sample	$\delta^{13}\text{C}_{\text{V-PDB}}$ [‰] s.d.: 0.5‰	$\delta^2\text{H}_{\text{V-SMOW}}$ [‰] s.d.: 15‰	$\delta^{18}\text{O}_{\text{V-SMOW}}$ [‰] s.d.: 1.2‰
East Indian sandalwood oil Mysore (A)	-29.6	-168	12.2
East Indian sandalwood oil Mysore (B)	-28.4	-193	11.6
East Indian sandalwood oil BM (A)	-28.1	-194	9.2
East Indian sandalwood oil BM (B)	-28.3	-158	12.3
East Indian sandalwood oil BM (C)	-27.9	-158	12.7
East Indian sandalwood oil BM (D)	-29.1	-166	13.8
East Indian sandalwood oil BM (E)	-28.9	-182	13.1
sandalwood oil (Tahiti)	-26.7	-133	12.1
<i>Santalum</i> <i>austrocaledonicum</i> (A)	-28.4	-197	10.7
<i>Santalum</i> <i>austrocaledonicum</i> (B)	-28.6	-195	11.0
<i>Santalum</i> <i>austrocaledonicum</i> (C)	-28.6	-187	10.1
<i>Santalum spicatum</i> (Australia)	-25.9 \pm 2.5	-245 \pm 40	13.9 \pm 3.5

Ylang-Ylang and Cananga Oils

The oils are obtained by steam distillation of the flowers of the tree *Cananga odorata* (Lam.) Hook. f. et Thomson, *forma genuina*, belonging to the family Annonaceae. The maturity of the flowers has a marked influence on the quality of the oils; the best oils were obtained when distilling fresh, fully mature flowers (41). Most likely this tree is native to the Philippines. Nowadays it is cultivated throughout tropical Asia and some islands of the Indian Ocean, mainly on Madagascar, the Comoro islands, and Nossi Bé on a domestic and an industrial scale. The world production is around 100 metric tons. The oil of ylang-ylang is one of the most important essential oils used in perfumery (42).

In general, four grades of the oil are commercially available: Extra, First, Second, and Third. In many cases also a fifth grade named Extra Super (43) is offered. In contrast to other essential oils these qualities are fractions, which are separated according to density and ester value (44, 45). In the first fractions oxygenated substances like e.g., linalool, *p*-cresyl methyl ether, methyl benzoate and benzyl acetate are responsible for the typical ylang-ylang odor. With increasing distillation time the concentration of these substances decreases whereas the concentration of sesquiterpene hydrocarbons like caryophyllene, germacrene D, (*E,E*)-farnesene etc. increases. Ylang-ylang oils are characterized by a diffusive, sweet, flowery, narcotic odor with fruity, balsamic and medicinal notes. The qualities Extra super, Extra and First are mostly used in fine fragrances. In addition, the oil is registered under FEMA number 3119 as natural flavor material. Main applications are ice cream, candy, chewing gum, and baked goods (46).

The essential oil of cananga is closely related to ylang-ylang oil. It is obtained by steam distillation of the flowers of *Cananga odorata* (Lam.) Hook. f. et Thomson, *forma macrophylla* (47). The oil is a light to dark yellow liquid with a characteristic, floral, slightly woody odor. Its quantitative composition resembles that of ylang-ylang Third grade oils, but is distinguished by its higher caryophyllene content (30 - 40%). The oil originates mostly from Java and from the Comoro and Réunion islands. The annual production is reported to be between 50 and 100 metric tons. It is mainly used in soap perfumes. The prices of the different ylang-ylang and cananga oil qualities ranged from about 150 US-\$/kg for the extra quality and 35 to 45 US-\$/kg for cananga oils in 2004. The sensorial important ingredients like *p*-cresyl methyl ether, linalool, methyl benzoate and benzyl acetate occur in higher concentrations in the more expensive oils and are easily available for low prices from synthetic origin; *p*-cresyl methyl ether 6 US-\$/kg and benzyl acetate 2 US-\$/kg in 2004. Therefore this study was focused on the addition of synthetic aroma-active substances and the differentiation of authentic ylang-ylang oils from cananga oils.

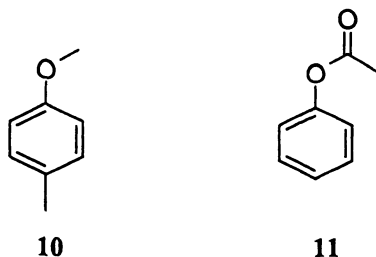


Figure 6: The chemical structures of *p*-cresyl methyl ether (10) and benzyl acetate (11).

In Table VII the GC/TC-IRMS analysis of *p*-cresyl methyl ether (10) showed a broad distribution of $\delta^2\text{H}_{\text{V-SMOW}}$ data from -148% to $+12\%$, while

Table VII: GC/C-IRMS and GC/TC-IRMS data of *p*-cresyl methyl ether (10) in ylang-ylang and cananga oils and of a commercial quality.

Sample	$\delta^{13}\text{C}_{\text{V-PDB}} [\text{‰}]$ s.d.: 0.4-1.5‰	$\delta^2\text{H}_{\text{V-SMOW}} [\text{‰}]$ s.d.: 10-12‰	$\delta^{18}\text{O}_{\text{V-SMOW}} [\text{‰}]$ s.d.: 1.2-2.0‰
<i>p</i> -cresyl methyl ether comm. (ex Cananga)	-33.1	-60	15.4
<i>p</i> -cresyl methyl ether comm. (ex Ylang II)	-32.2	-148	11.4
<i>p</i> -cresyl methyl ether comm. (ex Ylang I)	-33.2	-34	11.9
<i>p</i> -cresyl methyl ether comm. (ex Ylang FF)	-33.8	12	11.4
<i>p</i> -cresyl methyl ether comm. (ex Ylang)	-32.9	-39	13.1
<i>p</i> -cresyl methyl ether synth.	-31.2	-180	9.6

$\delta^{13}\text{C}_{\text{V-PDB}}$ values between -33.8‰ and -32.2‰ and $\delta^{18}\text{O}_{\text{V-SMOW}}$ data between 11.4‰ and 15.4‰ are in a fairly narrow window. In addition the $\delta^2\text{H}_{\text{V-SMOW}}$ data are remarkably shifted towards positive values in comparison to other essential oils, which might be an effect of the above-mentioned fractionation procedure. Synthetic *p*-cresyl methyl ether (10) shows a $\delta^2\text{H}_{\text{V-SMOW}}$ value of -180‰ , which is easily distinguished from ylang-ylang oil fractions and cananga oil with $\delta^2\text{H}_{\text{V-SMOW}} -60\text{‰}$.

The situation appears different for benzyl acetate (11), which is shown in Table VIII. For a synthetic quality $\delta^2\text{H}_{\text{V-SMOW}}$ value of -88‰ was found in our own measurements and $+352\text{‰}$ in the literature (48). In this case the $\delta^2\text{H}_{\text{V-SMOW}}$ data -191‰ to -197‰ of benzyl acetate (11) from the authentic oil qualities are more negative with a clear separation from synthetic material. The investigation of additional compounds like linalool, β -caryophyllene, germacrene D, geraniol, cinnamyl acetate, α -cadinol, benzyl benzoate, methyl benzoate and benzyl salicylate did not add additional points of difference.

Experimental mixtures of *p*-cresyl methyl ether (10) in the range from 3.9% to 5.3% and benzyl acetate (11) in the range from 4.7% to 10.9% with the corresponding oils show a significant shift for C, H, O data of the analyzed oils. Based on the data of this study differentiation of authentic oils and blended qualities seems to be possible via GC/TC-IRMS. In order to complete the picture additional measurements of commercial samples of different origin are necessary.

The analysis of trace compounds revealed the presence of mint sulfide, 2-phenylnitroethane and benzyl cyanide. The later 2 compounds are very powerful aroma chemicals, which have been identified in ylang-ylang oils (41) and several flowers (49). Buttery and coworkers identified 2-phenylnitroethane in tomatoes and described the formation as a variation of the biosynthesis of cyanogenic glycosides (50). Our own data show the occurrence of 2-phenylnitroethane and benzyl cyanide in the headspace of canola (*Brassica napus*) with a relative concentration of about 3.2% and of *Hemerocallis* spec. with 1.2%.

Conclusions

This study illustrates the capabilities of modern techniques like GC/MS, enantioselective gas chromatography, S- and N-selective detection and isotope ratio mass spectrometry (IRMS) for the authenticity control of four essential oils oregano oil, laurel leaf oil, sandalwood oil and ylang-ylang oil.

13 oregano oils comprising commercial qualities as well as self prepared samples were analyzed via GC/C-IRMS and GC/TC-IRMS regarding the addition of synthetic carvacrol and thymol. For both compounds differentiation of specific synthetic qualities has been achieved by IRMS data.

Table VIII: GC/C-IRMS and GC/TC-IRMS data of benzyl acetate (11) in ylang-ylang oils and of a synthetic quality.

Sample	$\delta^{13}\text{C}_{\text{V-PDB}}$ [‰]	$\delta^2\text{H}_{\text{V-SMOW}}$ [‰]	$\delta^{18}\text{O}_{\text{V-SMOW}}$ [‰]
	s.d.: 0.4-1.7‰	s.d.: 10-16‰	s.d.: 0.6-1.0‰
benzyl acetate comm. (ex Ylang II)	-32.9	-194	13.2
benzyl acetate comm. (ex Ylang I)	-31.7	-191	11.7
benzyl acetate comm. (ex Ylang FF)	-32.2	-197	14.5
benzyl acetate comm. (ex Ylang)	-31.6	-187	14.6
benzyl acetate synth.	-31.3	-88	7.4

Four laurel leaf oils were subjected to chiral analysis of α -terpinyl acetate and related by-products. In addition the C, H, O stable isotope pattern of α -terpinyl acetate and eugenol methyl ether in oil samples and synthetic qualities was determined. Both techniques enantioselective-GC as well as GC/TC-IRMS provided excellent data for the investigation of blends with synthetic material.

Twelve sandalwood oil samples from *Santalum album*, *Santalum austrocaledonicum*, and *Santalum spicatum* were checked via GC/MS analysis of key compounds and GC/C-IRMS- and GC/TC-IRMS-analysis of (+)-*cis*- α -santalol (7), (*Z*)- α -*trans*-bergamotol, (-)-*cis*- β -santalol (8), epi- β -santalol, (-)-*cis*-lanceol (9) and *cis*-nuciferol. Based on $\delta^{13}\text{C}_{\text{V-PDB}}$ and $\delta^2\text{H}_{\text{V-SMOW}}$ data, the sample of sandalwood oil ex Tahiti (*Santalum album* var. *marchionense*) could be differentiated from other investigated qualities. The ratio *cis*- α -santalol/*cis*-lanceol and published data on the distribution of β -bisabolol isomers in the 3 sandalwood oil representatives (37), provided additional criteria for differentiation.

In summary, this study explains the possibilities of modern authenticity control for essential oils. At the same time latest data on four important essential oils are provided.

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Chapter 7

Quality Control of Olive Oil: Analytical and Organoleptic Schemes for Quality Grading and Purity Control of Olive Oil

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This chapter gives an overview of international standards and EU legislation to evaluate the quality and purity of olive oil. The presented analytical methods and specified limits for chemical parameters are being applied by government and private food surveillance laboratories as well as by the olive oil business for quality control in each stage of the supply chain – from the olive mill to the supermarket shelves. To be accepted by regulatory bodies and trade partners the laboratories need to be accredited by the International Olive Oil Council (IOOC). The same applies for the organoleptic assessment of virgin olive oils which has to be performed by IOOC approved panels in a highly standardized manner.

Introduction

Olive oil is an integral component of the traditional Mediterranean diet which has been associated with a long life in good health and a reduced risk of several chronic diseases as compared to Western diets (1). Since November 2004 the US Food and Drug Administration has allowed a qualified health claim for olive oil stating "Limited and not conclusive scientific evidence suggests that eating about two tablespoons (23 grams) of olive oil daily may reduce the risk of coronary heart disease due to the monounsaturated fat in olive oil..."

In addition to its healthiness, its perceived naturalness and tastiness led to the growing popularity of olive oil, also and in particular outside the Mediterranean region where it has been produced and consumed already for centuries. More than 90% of the world production (2-2.5 million tons) is still taking place in the Mediterranean countries (2). Among the emerging producing countries are Argentina, South Africa and the United States. In the U.S. olive oil is almost entirely produced in California (Central Valley and North Sacramento Valley).

Production of Olive Oil

Compared to other vegetable oils, olive oil is relatively expensive. This is due to the significant proportion of manual work still required for growing, harvesting and milling of good quality olives and the decentralized infrastructure with a huge number of small orchards and mills (2). To obtain olive oil of excellent quality, only undamaged olive fruits should be milled, within 24 hours after harvesting. The fruits are washed and crushed to a paste that is subsequently kneaded with lukewarm water to facilitate coalescence of oil droplets (malaxation). Separation of olive oil and olive pomace is achieved by pressing, centrifugation or decanting. Only mechanical or other physical processes and ambient temperatures are allowed during olive oil production in order to protect the oil properties. After milling, virgin olive oil may only undergo the following treatments: washing, filtration, centrifugation, decantation.

Standards and Legislation regulating Olive Oil

The permitted production processes and oil treatments are defined in the Trade Standard Applying to Olive Oils and Olive Pomace Oils of the IOOC (3). In these standards, the grading and quality control of olive oil are defined as well as the analytical methods to be used. The IOOC standard is equivalent to the

Draft Revised Standard for Olive Oils and Olive Pomace Oils (At step 8 of the Procedure) of the Codex Alimentarius Commission (4). The European Union has adopted the IOOC standards into its legislation. The European situation is presented in this overview. If differences exist compared to the IOOC/Codex standards it is mentioned in the text.

In the USA (which has observer status in the IOOC) still the USDA Standards for Grades of Olive Oil from 1948 (5) are valid defining the quality grades “fancy”, “choice”, “standard” and “substandard”. The standards are difficult to enforce because only few measurable parameters are defined (free fatty acids, defects such as cloudiness/sediment, odor and flavor) and no methods are specified. Furthermore, imported olive oils are graded according to the IOOC system and also many domestic producers and importers are committed to and voluntarily follow the internationally accepted standards. Consequently, the Californian Olive Oil Council has submitted a petition to the USDA’s Agricultural Marketing Service in 2004 to adopt the IOOC standards in the U.S. as well (6). The petition is supported by the U.S. olive oil business.

Quality grading of olive oil

In the EU, eight grades of olive oil exist of which four may be sold to consumers (Table I). The IOOC defines a 9th grade of olive oil (ordinary virgin olive oil) with a quality in-between lampante and virgin. This olive oil can be legally marketed in countries that permit it. The EU allowed this grade of olive oil until October 2003 (7-9). Also refined olive oil is legally traded in some countries. In the EU virgin olive oil must be added to the bland refined olive oil to add back some of the characteristic olive oil taste. A ratio between refined and virgin olive oil is not defined and depends on the local taste preference. On the EU retail shelves, only extra virgin olive oil and blended olive oil are commonly found. This overview will therefore focus on these two olive oil categories.

How to determine the quality grade of an olive oil sample is laid down in EU regulation 2568/91 (10). In Annexes I to XIX of this regulation, the legal limits for numerous chemical parameters are defined for the different olive oil categories. Also the sampling procedure and analytical methods are specified there. The latest amendments of the regulation are EU regulations 796/2002 (11) and 1989/2003 (12). The criteria and officially accepted methods available for quality grading and purity control of olive oil will be briefly presented in the following paragraphs. Citations of suitable IUPAC, AOCS or ISO methods can be found in the IOOC and Codex Standards (3,4).

Next to updates of a few legal limits and the sampling procedure in order to harmonize the values with the IOOC standards, regulation 1989/2003 also adjusts the evaluation scheme to only eight olive oil categories. Furthermore, it

proposes decision trees for verifying whether an olive oil sample is consistent with the category declared. The decision trees are not mandatory in terms of sequence but aim at minimizing analytical effort. The example for extra virgin olive oil is given in Figure 1. All values specified are applicable until the end of the oil's shelflife and they already include analytical errors. Thus, no tolerance based on repeatability or reproducibility is permitted. Comparable decision trees exist for the other olive oil grades. Unique to extra virgin and virgin olive oil, however, is the organoleptic assessment.

Table I. Quality grades of olive oil and olive pomace oil

<i>Grade</i>	<i>Description</i>	<i>May be retailed in EU</i>
Extra virgin olive oil	superior category olive oil obtained directly from olives and solely by mechanical means	yes
Virgin olive oil	olive oil obtained directly from olives and solely by mechanical means	yes
Lampante olive oil	crude olive oil with quality defects, needs to undergo refining before consumption	no
Refined olive oil	olive oils that have undergone refining	no
Blended olive oil	composed of refined olive oils and virgin olive oils	yes
Crude olive-pomace oil	obtained by solvent extraction or 2 nd centrifugation from press residue, needs to undergo refining before consumption	no
Refined olive-pomace oil	olive pomace oils that have undergone refining	no
Olive-pomace oil	composed of refined olive pomace oil and virgin olive oil	yes

SOURCE: Grades and descriptions taken from References 7-9.

Markers for Hydrolysis

The acidity is determined by analyzing the free fatty acids (FFA) by titration with ethanolic potassium hydroxide (10). FFA are already formed by enzymatic hydrolysis of the triacylglycerols (TAG) during maturation of the olive fruit. Elevated FFA values indicate accelerated hydrolysis, e.g. due to improper storage of olive fruits or too extensive malaxation. In the EU an olive oil would

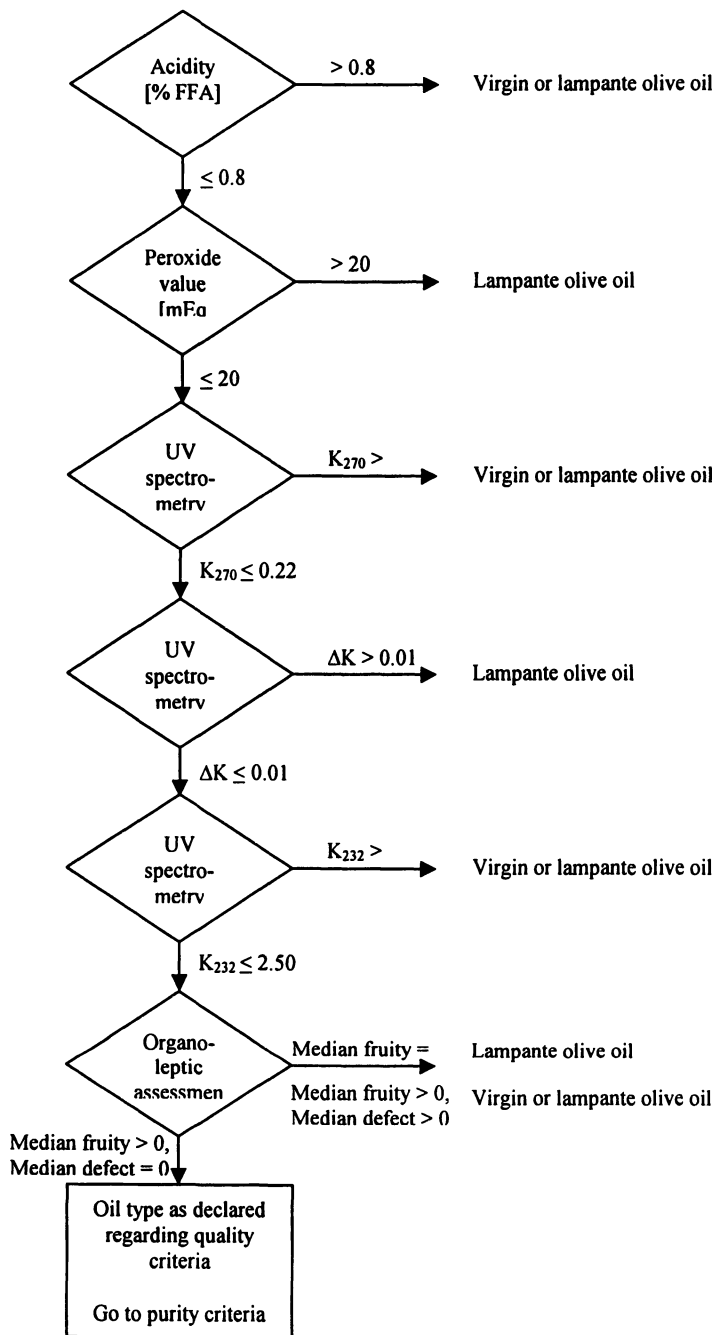


Figure 1. Decision tree to determine if an oil sample declared as extra virgin olive oil conforms with the respective quality criteria (12)

be classified as lampante if FFA exceed 2.0%. In the IOOC standards the upper limit is 3.3% (between 2.0 and 3.3% FFA the oil would be ordinary virgin). For blended olive oil the limit is 1.0% FFA.

Markers for Oxidation

The peroxide value (POV) is determined by titration (10). During oil storage, the level of peroxides increases depending on storage conditions (light, temperature, time, oxygen exposure) followed by a degradation of the hydroperoxides to secondary oxidation products among which are undesirable aroma compounds. Due to its fatty acid composition and natural antioxidant system olive oil is remarkably stable. If kept closed in the dark at approx. 10-15 °C, olive oil can easily have a shelflife of two years. All virgin olive oils suitable for consumption need to have a $POV \leq 20$ milliequivalents (mEq) of active oxygen/kg, blended olive oil a $POV \leq 15$ mEq O_2 /kg.

To determine the specific UV absorption (K) at 232 and 270 nm olive oil is dissolved in iso-octane (10). UV light of these wavelengths is absorbed by primary and secondary oxidation products such as conjugated hydroperoxides, aldehydes, ketones and conjugated dienes and trienes, respectively. This parameter is thus an additional indicator for the oxidative status and freshness of an oil. The K_{232} and K_{270} values permitted for virgin olive oil are only slightly higher than those for extra virgin (Figure 1): 2.60 and 0.25 respectively. Blended olive oil may have higher K_{270} and ΔK (0.90 and 0.15, respectively), K_{232} is not specified.

Organoleptic assessment

Since September 2002 the EU has adopted the new standardized IOOC method for organoleptic assessment of virgin olive oils (11). To make this assessment as objective as possible, the IOOC has specified the screening, selection and training of suitable panel members, the panel room and test glasses, the vocabulary and the statistical evaluation of the results (13). First, each of the 8-12 panel members evaluates the presence of organoleptic defects. If a defect is detected it is scored on an open scale ranging from 1-10. The six most common defects are explicitly mentioned on the panel sheet. They are caused by improper storage of olive fruits or olive oil (Table II). Ten other defects are specified in the regulation (11). Also positive, i.e. characteristic, attributes are defined (Table II). After having checked for defects, each panelist scores the intensity of the positive attributes fruity, bitter and pungent.

Table II. Main organoleptic attributes of virgin olive oil

<i>Organoleptic attribute</i>	<i>Definition</i>
Defects:	
Fusty (atrojedo)	characteristic flavor of oil from piled olives in advanced anaerobic fermentation
Musty (humidity)	characteristic flavor of oil from olives in which large numbers of fungi and yeasts had developed as a result of storage for several days in humid conditions
Muddy sediment	characteristic flavor of oil that has remained in contact with sediments in vats and tanks
Winey/vinegary	characteristic flavor of oils reminiscent of wine or vinegar, due basically to formation of acetic acid, ethyl acetate and ethanol by fermentation of olives
Metallic	flavor reminiscent of metal, characteristic of oil that has been in prolonged contact with metal surfaces during crushing, mixing, pressing or storage
Rancid	flavor of oil that has become oxidized
Positive attributes:	
Fruity	range of smells (depending on variety) characteristic of oil from healthy fresh fruit, green or ripe, perceived directly or retronasally
Bitter	characteristic taste of oil from green olives or olives turning color
Pungent	tingling sensation characteristic of oil made at the beginning of the season mainly from olives that are still green

SOURCE: Definitions taken from References 11 and 13.

The panel supervisor calculates the median and robust coefficient of variation (CV) of the attributes. If the robust CV exceeds 20% the panel evaluation needs to be repeated. For quality grading of an oil sample only the median of the main defect and the fruitiness are relevant as can be seen from Figure 1. In countries where the ordinary virgin grade is used as well, olive oils with a main defect between 2.5 and 6.0 or with a defect ≤ 2.5 and absent fruitiness are still considered fit for consumption and do not need to undergo refining as in the EU. The EU deliberately chose a stricter grading scheme since November 2003 to allow only high quality virgin olive oils on the market (8, 11).

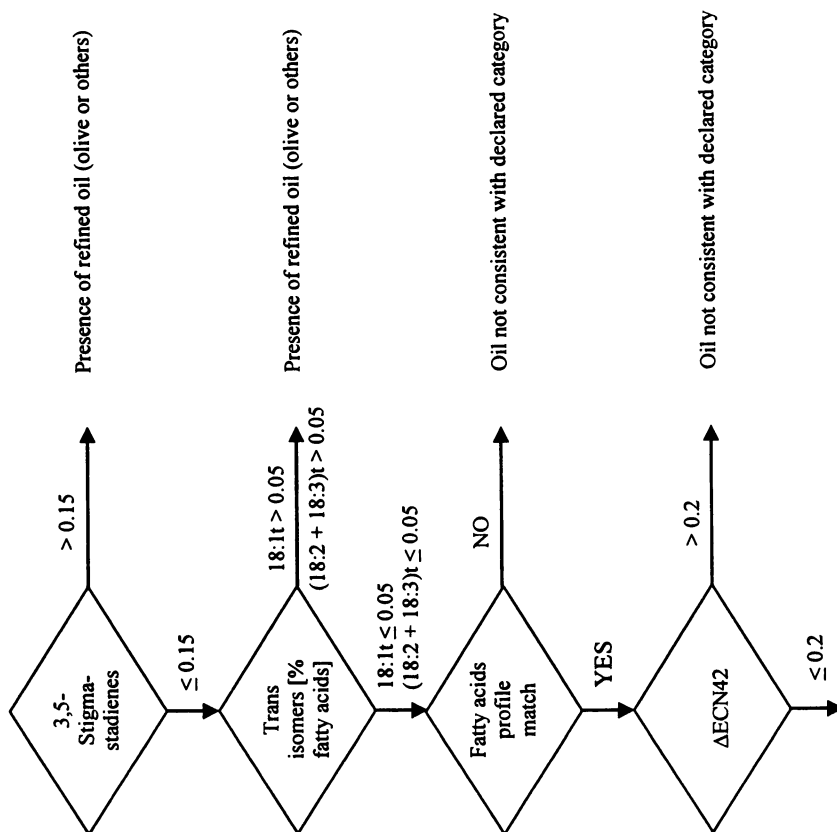
Purity control of olive oil

After having checked the quality parameters the oil sample would be analyzed for purity criteria. Also for purity control the proposed analytical methods are mainly ISO, IUPAC or AOCS methods and need to be performed by accredited laboratories (3,4). Again decision trees are proposed in the EU regulations that can be followed to detect different attempts of adulteration (12). The example for extra virgin and virgin olive oil is given in Figure 2. Comparable decision trees exist for the other olive oil grades. For blended olive oil and olive pomace oils, a check for the presence of esterified oils is also specified. Esterified oils are obtained by re-esterification of olive free fatty acids and glycerol. They can be detected by analyzing the percentage of saturated fatty acids (C 16:0 + C 18:0) in the 2-position of the TAG. The official method suggests gas chromatography after enzymatic hydrolysis of the TAG to 2-monoacylglycerols. The maximum value for blended olive oil is 1.8% (for virgin olive oil 1.3%). A blank value is required because lipases contain a small amount of C 16:0, which leads to systematically elevated values (14).

Markers for Presence of Refined Oils

The most suitable marker to date to detect the presence of refined (olive) oil in virgin olive oil is stigmasta-3,5-diene. It is formed by dehydration of β -sitosterol during the bleaching step and increases with increasing temperature and increasing concentration of bleaching earth (15). Virgin olive oil contains < 0.1 mg/kg stigmastadiene, fresh oil measured directly after milling even < 0.05 mg/kg. The maximum limit for virgin olive oil has been set at ≤ 0.15 mg/kg. This limit already includes unintended contamination with refined olive oil and may therefore in no instance be exceeded. The determination is performed by capillary gas chromatography after isolation of unsaponifiable matter and fractionation of the steroids on silica gel (16).

To detect thermally treated (olive) oil in virgin olive oil the sample is analyzed for trans fatty acids (TFA). The isomerization of *cis* unsaturated fatty acids to their *trans* isomers increases with increasing deodorization (refining) temperature (17). The limit for *trans* isomers of mono- (C 18:0) and polyunsaturated fatty acids (C 18:2 + C 18:3) has been set at 0.05% each for virgin olive oils and at 0.2% and 0.3%, respectively, for blended olive oil. TFA are analyzed by gas chromatography on polar columns after interesterification with cold methanolic potassium hydroxide (11).



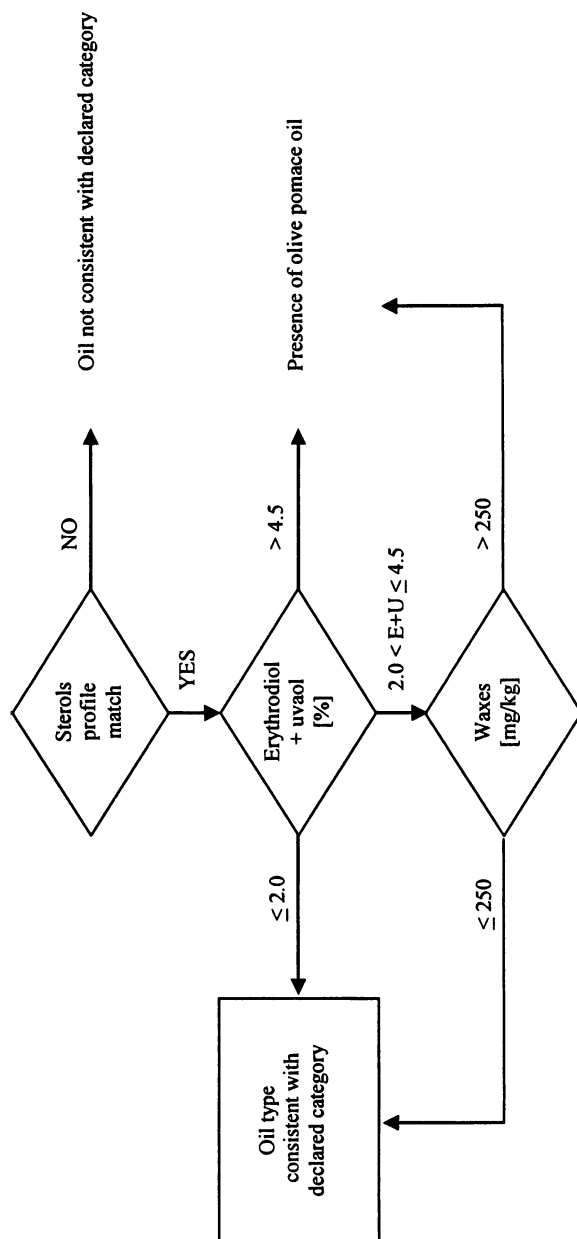


Figure 2. Decision tree to determine if an oil sample declared as extra virgin or virgin olive oil conforms with the respective purity criteria (12)

Markers for the Presence of Other Animal or Vegetable Oils

While analyzing TFA the complete fatty acid profile can be determined. The fatty acid composition is a characteristic property for any oil or fat. Olive oil is characterized by very low levels of minor fatty acids. Limits have therefore been set for myristic (0.05%), linolenic (1.0%), arachidic (0.06%), eicosenoic (0.4%), behenic (0.2%) and lignoceric (0.2%) acid. The limit for linolenic acid is still under review as some olive varieties seem to have higher levels than the traditional Mediterranean varieties (6).

As some vegetable oils have a very similar fatty acid composition as olive oil, a second method to detect seed oils based on differences in the TAG profile is proposed. From the fatty acid profile the theoretical TAG profile can be calculated according to the methodology specified in the regulation (18). This value can be compared with the TAG value obtained by HPLC determination (after silica gel clean up). If the difference between the measured and the calculated Equivalent Carbon Number with molecular mass of 42 (ECN42) exceeds 0.2 in case of virgin olive oil or 0.3 in case of blended olive oil the sample has most likely been adulterated with a seed oil or hazelnut oil.

Also minor oil components can help to detect the presence of other oils in olive oil. Particularly characteristic is the sterol profile. Olive oil contains minimum 93.0% β -sitosterol (note: peak also comprises $\Delta 5,23$ and $\Delta 5,24$ -stigmastadienol, chlerosterol, sitostanol and $\Delta 5$ -avenasterol). Elevated levels of other sterols exceeding the specified limits indicate the presence of foreign oils and fats: >0.5% cholesterol (animal fat), > 0.1% brassicasterol (rapeseed oil), > 4.0% campesterol, > 0.5% $\Delta 7$ -stigmastenol. If stigmasterol > campesterol the presence of bean oil is likely. The sterol profile is analyzed by capillary gas chromatography (10). The minimum total sterol content of olive oil is 1000 mg/kg. In olive pomace oil even higher amounts can be expected.

Approaches to Detect Small Amounts of Hazelnut Oil

Fatty acid composition and sterol profile of hazelnut and olive oil are very similar. With the official EU methods, the addition of small amounts of hazelnut oil to lampante olive oil is therefore very difficult to detect. In order to identify a method that is suitable to detect an addition of <10% hazelnut oil, the EU has funded an international project to compare and evaluate different analytical and statistical approaches (19). Among the most promising methods are the chromatographic analysis of major (TAG structural differences) or minor components (such as sterols or filbertone), spectroscopic methods such as ^1H and ^{13}C Nuclear Magnetic Resonance (NMR), Fourier transform-mid-infrared (FT-MIR), FT-Raman, and isotopic methods such as elemental analysis pyrolysis

isotopic ratio mass spectrometry of ^2H (^2H -EA-Py-IRMS), particularly if these methods are combined with multivariate statistical analyses or artificial neural networks.

Until a suitable method has been incorporated into the catalogue of official analytical methods, the IOOC recommends the method by Fedeli which calculates and measures ECN42 and ECN44 with HPLC (20). If the relative difference between theoretical and measured ECN42 ($\Delta_{\text{rel}}\text{ECN42}$) is negative or exceeds 14.88% and if the ratio between $\Delta_{\text{rel}}\text{ECN44}$ and $\Delta_{\text{rel}}\text{ECN42}$ exceeds 0.90 an addition of hazelnut oil is most likely (21). The method is said to detect additions of 3-10% hazelnut oil. For confirmation the IOOC recommends to analyze the profile of esterified sterols according to the method by Mariani et al. (22).

Markers for the Presence of Olive Pomace Oil

Elevated amounts of the two hydroxytriterpenes erythrodiol (3 β ,28-dihydroxy olean-12-en) and uvaol (3 β ,28-dihydroxy urs-12-en) that can be analyzed together with the sterols (10) are an indication that olive pomace oil is present in the sample. These compounds occur naturally on the skin of the olive fruit. Solvent extraction or second pressing are more effective means to extract these compounds into the oil. If the values exceed 4.5% of total sterols the olive oil is considered adulterated. Values between 2.0-4.5% need additional confirmation by analysis of waxes (Figure 2). Waxes are esters of long chain fatty acids with long chain fatty alcohols. They protect the skin of the olive fruit and are therefore more abundant in olive pomace oil (≤ 350 mg/kg) than in virgin olive oil (≤ 250 mg/kg). Waxes can also be formed during storage and/or refining. Therefore limits for lampante (≤ 300 mg/kg) and blended olive oil (≤ 350 mg/kg) are higher. Analysis of waxes (C40-C46) is performed by capillary gas chromatography after isolation on silica columns (23).

Other Quality Relevant Parameters

Also belonging to the quality control of olive oil, although not relevant for grading and labeling, are analyses for contaminants to check if the sample is complying with Codex standards or other EU or national legislation (e.g. with regard to pesticides, polyaromatic hydrocarbons, aromatic solvents, heavy metals). In the olive oil standards maximum limits for the presence of halogenated solvents (0.1 mg/kg of any single solvent, 0.2 mg/kg of total halogenated solvents), trace metals (3 mg/kg iron, 0.1 mg/kg copper), heavy

metals (0.1 mg/kg lead, 0.1 mg/kg arsenic), moisture and volatile matter (0.2%) and impurities (0.1%) have been specified (3,12).

Outlook

The methods offered by the IOOC and the EU provide a comprehensive set of tools to evaluate the quality and purity of olive oil. It needs to be acknowledged, however, that official methods do not always reflect the latest state-of-the-art in analytical chemistry and chemometrics. Before new emerging methods are sufficiently validated and incorporated into international standards they are already used by specialized laboratories for quality control. However, they do not suffice in cases of legal dispute. The progress in analytical and statistical methods will not only improve the existing methods but will most certainly add further aspects of interest to the official evaluation scheme in the future. Active fields in the scientific literature are, among others, the search for analytical approaches to support the organoleptic assessment, to characterize the profile of minor components and to detect very mild but unlawful treatments of virgin olive oil.

Flavor Analysis

The profile of volatile flavor compounds is characteristic for virgin olive oils and depends on olive cultivar, ripeness, climatic conditions etc. The compounds are mainly produced by biogenic pathways. Virgin olive oil of lower quality, however, possesses a more complex profile, because additional volatiles are formed as a result of olive over-ripening, action of bacteria and moulds, or oxidation of the oil during storage. Several attempts have been made to relate the profile of volatiles to the sensory defects listed in Table II and identify those compounds responsible for each individual defect (24 and references cited therein). Due to the lack of accuracy and ability to integrate the overall organoleptic perception, the instrumental approach can hardly fully replace an experienced sensory panel, however, it can be considered as a tool for e.g. rough pre-elimination of samples with defects.

Phenol Analysis

Virgin olive oil contains a plethora of minor components, of which polar phenolics deserve a special attention, as their occurrence in olive fruit and oil is unique, and as they have been associated with some of the beneficial health

effects (25). They also contribute to the unique taste of virgin olive oil and together with the monounsaturated fatty acids they are responsible for the remarkable oxidative stability of olive oil (24). The polar phenolic fraction of virgin olive oil consists of a mixture of compounds (simple phenols, secoiridoid aglycones, lignans, flavones, etc.), each of which vary in chemical properties and impact on the oil quality (26). Application of modern chromatographic methods within last decade have enabled a routine characterization of the profile of major phenolics in virgin olive oil. Whilst the total amount of phenolics is a subject of large natural variation (27) (depending mainly on climatic and agronomic conditions, the degree of maturity, and technology of olive processing), the phenolic profile can be used as a fingerprint that can provide additional information on olive variety or specific taste attributes (28,29). Combined with a comprehensive understanding of reactions of phenolics during storage, the phenolic profile can also serve as an indicator of hydrolytic (proportion aglycons/simple phenols) (30) and, in particular, oxidative (proportion mono-*o*-diphenols) changes during storage (31).

Mild treatment of virgin olive oil

Undesirable flavor compounds could be removed from lower quality virgin olive oil by vacuum distillation at temperatures below those required for the formation of TFA or stigmastadiene. An approach to detect such mild but unlawful “upgrading” of olive oil is the analysis of chlorophyll degradation products (32). It appears that the formation of pyropheophytine, which also happens during oil storage, is considerably accelerated already at moderately elevated temperatures.

Amounts of free fatty acids slightly exceeding the legal limits could be removed by chemical neutralization followed by mild deodorization. This treatment seems to increase the isomerization of the 1,2-diacylglycerides (DAG) to 1,3-DAG (33). Both methods are currently explored in quality control with first promising results (34).

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Chapter 8

Fish and Seafood Authentication

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This chapter deals with the problem of fish species identification. A brief overview about the possible difficulties behind the correct labelling of seafood products is offered. Substitution of cheaper species for expensive or appreciated ones can be a common practice, especially when morphological features are removed. When external species markers are removed, other characters should be used to identify species, and these are the biochemical species markers, mainly proteins and nucleic acids. The use of biomolecules as species markers is presented and the advantages and disadvantages of using each of them are discussed. Finally, it contains what has been done in the field of species identification using biochemical markers presenting in more depth the developments made using DNA as species markers, and several DNA techniques used for solving the problem of species identification are presented.

The Problem of Fish Authentication

Fish is an important part of the human diet; it now represents an average of 15.4% of animal protein consumption and 5.5% of total protein consumption (1). Fish is consumed in different amounts and ways around the world, and these data may change substantially depending on the world region. In Western Europe the average per capita consumption in 1995 was 23 kg/person/year and 22 in North America while in undeveloped regions such as Africa or South America these values fell to 7-8 kg/person/year.

Fish authentication is important to developed countries where the fish supply is large and special attention is paid to the type of fish which is consumed. This is especially important in countries with a large fish consumption tradition such as Japan or Mediterranean countries. Usually the problem is the shortage of some traditional species, due to the high demand for these species, which makes the substitution with cheaper species profitable.

As an example, not long ago most European countries consumed fish coming from their own fishing fleet in the nearby waters. A limited number of fish species was consumed at that time, and all were well known by all those involved in the whole chain from capture to distribution and consumers. Usually fish were landed as whole specimens, not even eviscerated, from one or several species, which were easy to recognize because of the presence of all morphological characteristics.

The situation has changed nowadays driven by a number of factors: development of high seas fishing vessels, improvement of elaboration and conservation processes, establishment of a modern fishing industry in some developing countries such as South American, African or Asian countries, the increase and improvement of worldwide aerial, maritime and terrestrial communications, and market globalization. All these factors have lead to an increase in the number of fish species, both fresh and processed, which are now present in our markets.

This extended consumption of new species has been accompanied by a continuous increase of total catches. FAO data (2) indicate that the actual global capture from fisheries (1998 data) is around 90 million tonnes per year, and that total capture had a five fold increase from 1950 to 1990, also increasing the number of species with commercial value. Fish consumption has followed the same path, human fish consumption has increased from 40 million tonnes in 1970 up to 86 million tonnes in 1998, with an increase of as much as 31% of fish consumption between 1990 and 1997. However, at the same time, decrease in the capture of most appreciated species like cod, sole, or European hake, has been reported, due mainly to overexploitation of resources. This means that they are being replaced by alternative species.

The price of each fish species or derived product is a function of several variables such as intrinsic quality, consumer acceptance or demand, resource availability, etc. Consumers should always be informed about the products they buy, so that a purchase decision can be made based on sufficient and truthful information. This also concerns the fish processing industry because they have to rely more and more, on pre-processed starting material (with variable prices depending on the quality and species). They also need to know the fish species involved as they are responsible for the labelling of their seafood products.

During the last decade, the European Union and other countries have been issuing food labelling normatives, including those specific for fish and seafood products. These normatives are more demanding about the information that should be included in the label such as commercial name, species or geographical origin. In order to control the compliance to these regulations, the administration should have the adequate tools to verify, among other requisites, the authenticity of species indicated on the label.

Biochemical Species Markers

The most quoted definition of species is that of Mayr (3), isolation species concept, and in this definition it is stated that “*species are groups of interbreeding natural populations that are reproductively isolated from other such group.*”

Identification of a particular fish species can be made by using a number of taxonomical morphological characters (“*any attribute of a member of a taxon by which it differs or may differ from a member of a different taxon*”) such as skin patterns, body shape and size, shape and number of fins, eyes or even internal organs. Diagnostic characters are those taxonomic characters that uniquely specify a particular taxon (4). However, even when all morphological characters are intact and when dealing with very closely related species, identification of some fish species is rather difficult. These morphological characters are removed when fish is processed (beheaded, eviscerated, skinned, filleted, smoked, cooked or canned) making identification using these characters very difficult or even impossible. Because of these difficulties, it is evident that there is a need for alternative methods of species identification.

Proteins have been widely used as species markers, not only for identification of animals for human consumption, but for taxonomic purposes (4). Early works on development of species identification techniques showed that separation of water soluble proteins by electrophoresis could be used for this purpose (5, 6). Later, electrophoretic techniques were improved with the development of isoelectric focusing, and well resolved water soluble protein patterns were obtained which permitted the differentiation of closely related fish

species (7). Some other techniques, based on the analysis of proteins, were described such as 2-D electrophoresis (8, 9), urea-IEF (10), SDS-PAGE (9, 11), capillary zone electrophoresis (12, 13), reverse-phase high performance liquid chromatography (14, 15, 16) and immunological techniques (17, 18, 19, 20).

In summary, protein analysis has been widely employed in the past for fish species identification and it is useful in the case of fresh, refrigerated and frozen seafood. One of the advantages of the techniques, especially IEF, is that it is relatively cheap, allows quick processing time, and it does not require a highly trained staff, or sophisticated and expensive equipment. However, protein analysis has two major drawbacks: it is useless for heated seafood products, because of protein denaturation, and it has low resolution power which is problematic when closely related species need to be differentiated.

DNA sequence information has also been employed for species identification, especially after the spread of polymerase chain reaction (PCR) (21). PCR allows the amplification and analysis of selected fragments of DNA in a relatively short time (2 hours). The analysis of the sequence contained in the amplified product can be performed using different techniques, probably the widest used is sequencing. DNA sequences from both nuclear and mitochondrial DNA have been used for species identification and they have the following advantages:

- different DNA regions can be selected for analysis with different mutation rates and inter- and intraspecific variability, depending on the level of resolution required (individuals, population, species, genera, family, etc.)
- DNA is more stable than proteins to different industrial processes

DNA Methods for Fish Species Identification

The first requirement to identify species using DNA is to be able to extract DNA from the fish. The integrity of this DNA plays an important role and restricts the choice of analytical technique to be used. Although DNA is a very stable molecule, there is a number of factors involved in its degradation such as activity of nucleases (22, 23), oxidation, acidic treatment and thermal degradation (24). DNA is degraded to fragments smaller than 500 bp by temperatures higher than 100°C, depending on the temperature and time of the thermal treatment (25, 26, 27, 28). DNA extracted from sterilized products has an average size of DNA around 300 bp (29). Mitochondrial DNA should be the choice for species identification when dealing with heated products because of the higher number of copies per gram of tissue, compared with nuclear DNA, and the possible higher tolerance to heat of mtDNA than that showed by nuclear DNA (30).

Once DNA is extracted a second step usually involves the amplification of DNA by using the polymerase chain reaction (PCR) (31, 32). This reaction permits the synthesis of specific DNA fragments *in vitro*.

PCR requires knowledge of small stretches of DNA sequence flanking the fragment to be amplified; these are known as primers. A good primer design is of paramount importance for successful PCR and all derived techniques since the lack of perfect match between template DNA and 3' end of the primer might lead to a PCR failure (33). Cytochrome b has been most often used for species identification, although other gene fragments like DNA coding for ribosomal RNA, cytochrome oxidase, ATPase and control region have also been employed.

There are two methods to amplify DNA using a polymerase. In the first method non specific primers are employed, and that means that it is not necessary to know primers sequence. After amplification, a specific profile of bands may result and this can be used for species identification. The second method requires knowledge of primers sequence. In this case a particular DNA fragment is obtained and the amplified DNA is then analysed using other secondary analysis methods.

Random Amplification of Polymorphic DNA (RAPD)

The technique is very simple and requires the use of single and short arbitrary primers (9 to 10 mers) which anneal randomly at several locations of genomic DNA (34, 35). After amplification fragments of different sizes are obtained. These are separated using electrophoresis (agarose, polyacrylamide), and visualized (ethidium bromide or silver staining) resulting in specific patterns. These patterns can be used for species or population identification. However, it has some disadvantages: its high susceptibility to changes of DNA quality and/or quantity, the thermalcycler used for the amplification may influence the fragments obtained and also cycling conditions, polymerase, separation and detection conditions could also cause some alterations in the pattern, intraspecies variability may result in the obtainment of unspecific patterns. All of these parameters require standardization in order to obtain reproducible patterns (35, 36). Despite these problems, some authors have described RAPD methods that were reproducible (37) or that had been used for processed seafood or meat species identification (38).

Single Strand Conformation Polymorphism (SSCP)

SSCP is an electrophoretic technique that allows the rapid detection of mutations in a specific DNA fragment. The technique is based on the different

mobility of single strand DNA folding conformers (different sequence) that are subjected to an electric field under non denaturing conditions (39). The shorter the DNA fragment, in a specific range, the higher the resolution obtained with this technique. Usually optimal sizes range around 100-200 bp. In the highest resolution, differences in a single nucleotide are reflected in differences in electrophoretic mobility (40). Differences in mobility of single strands of certain DNA fragments can be species specific and several published works have demonstrated the usefulness of the technique for routine analysis of species (41, 42, 43).

Sequencing and Genetic Distance Measurement

The use of DNA sequencing for species identification was named as Forensically Informative Nucleotide Sequencing (FINS) by Bartlett and Davidson (44). The principle of the technique is to use the comparison of the DNA sequence from the product to be identified with a database of sequences of known species. The comparison is usually made using distance measurement algorithms to estimate the degree of similarity between the product and the known species sequences.

Commercial fish species groups comprise several species which can be genetically closely related. The best methods are those which rely on DNA sequence data bases of as many fish species as possible of a particular group.

One of the crucial parts in the development of an identification technique, based on sequence, is the selection of a target sequence. It should contain enough information to allow the differentiation of all the commercial fish species under study (i.e., gadoids). This means a high interspecific variability, though it should have as low as possible intraspecific variability (28). DNA fragment size will determine the application of the method to fresh, frozen, mildly heated or thermally treated products. Canned products should be analysed using DNA fragments no longer than 200 bp (28).

The use of DNA sequencing has several advantages with respect to other methods. Sequencing permits the classification of an unknown sample in the group of species to which it belongs (44, 45) and even more interestingly it also allows the detection of new species being put in the market or unusual species which may be sold under a particular label (46). It is also possible to overcome problems associated with the existence of intraspecific variability, since the whole sequence information is used to perform the identification.

Once the unknown sequence is obtained, the common method for identifying the species is to calculate a genetic distance among the unknown sequence and a set of reference sequences. Genetic distances can be then used to build a pairwise matrix of distance. The unknown sequence will show the lowest distance with the phylogenetic group to which it belongs (28).

Restriction Fragment Length Polymorphism (RFLP)

Sequence information, contained in a particular PCR fragment, is assayed by using restriction enzymes which recognize a very short sequence within the fragment. Usually the restriction enzymes used are four, or six nucleotides cutters, meaning that they would recognize a four or six nucleotide sequence and cut at different points before, within or past the recognized sequence. The resulting fragments are specific and generate a profile after separation using electrophoresis. The technique has been used for the identification of several fish species in processed seafood (5,27,28,30,44,47,48,49,50,51,52,54). However, its usefulness is questionable when a limited number of species is studied when developing the technique (55). The main problem is that there is no total guarantee that a previously unstudied species will produce a specific RFLP pattern; this means that it could give exactly the same pattern as some of the other species studied. Ideally, the number of species sequences used for selecting the diagnostic restriction enzymes should be those with potential to be used in a particular product. Unfortunately, the common situation is the substitution of one valuable species by a closely related (same family or same genus) species of a lower price. Usually identification is aimed to answer questions such as “which is the species included in this product?” If the method is devised only for the differentiation of two species, as it has been observed in some published works, for instance *Solea solea* and *Microchirus azevia*, one of the answers to the questions may be “this is not a sole”, only if the RFLP haplotype of the unknown sample is different from sole pattern. If the pattern is the same as sole, the answer should be “this might be sole but there is no total guarantee that this is sole.”

Specific PCR

Specific primers can be designed to permit the amplification of a species of interest and not in others. The presence of the PCR product is regarded as an identity proof. This technique has been employed for differentiating caviar species (56) and tuna species (57).

DNA Probes

DNA probes can be used in several formats. Hybridization of single strand DNA (ssDNA) from a particular species with ssDNA from an unknown has served as the basis for development of several identification methods. An early approach was the use of total genomic DNA as probe using a slot/blot assay (25, 58). However, this methodology did not work well with closely related species due to sequence homology producing cross-reactivity. Species specific satellite DNA oligonucleotide probes with no cross-reactivity were also tested for the identification of five animal species in a wide range of commercial products (59). The use of the probes permitted the semiquantitation of species down to a level of 2.5%.

Other methodologies have been investigated such as the use of PCR-ELISA which combines the specificity of the DNA hybridization with an ELISA detection of hybridized probes.

Another type of method, the so-called real-time PCR, is also a combination of hybridization and PCR. The method is relatively new and besides the ability of species identification also allows the possibility of quantification of species in a mixture.

An alternative to sequencing methods that is starting to show diagnostic applications is the use of high-density oligonucleotide probe arrays, commonly known as DNACHIPS. A DNA array, an elevated number of oligonucleotide probes, can be used to determine the identity of a target sequence by detecting specific nucleotides (60). For instance, 16000 bp sequence can be analysed by using 66000 probes (60). Probes are chemically attached to a solid substrate, the target can be labelled with a fluorescent reporter and then incubated with the array. If the target has a part of the sequence complementary to the probes in the array, a hybridization will take place. Hybridization strength will depend on the level of match between probes and target DNA. A system for measuring fluorescence scans the array and detects where the hybridization has occurred. Software processes the image obtained and correlates with particular probes being present at particular sites (61). The specific pattern is recognized by the software and gives an answer about the species being present in the unknown sample.

Conclusion

The objective of developing methods for species identification is to provide industry, control laboratories, administration or even consumers with methodologies which can be easily implemented in their laboratories. Therefore, the methodologies tend to be simplified as much as possible and, if the meat case

path is followed, there will be kits on the market for fish species identification as there are already for meat.

Examples of these kits are the “BioKits for Animal Speciation Testing” from Tepnel Biosystems Limited (UK), “Cooked Meat Species identification kit” from Cortecs Diagnostics, “DTEK™ Immunostick” from ELISA Technologies Inc. (USA). These methods are easy to perform and no special equipment is needed. Commercially available methods for fish species identification are based on sequencing (in some cases, access to a private database of sequences is given) or RFLP.

Commercial kits for identification of animal species based on DNA are already available. Some examples are “DNAAnimal BOS Ident” from Genescan (Scil Technology holding GmbH) and SureFood ID-Animal from Congen Biotechnologie GmbH (Germany). The methodology behind these kits is based on specific DNA probe hybridization and detection of hybridized probes with ELISA detection. However, up to now no commercial kits were found to identify commercial fish species.

Some companies are starting to show interest in the development of DNA chips such as the FoodExpert-ID developed by bioMérieux, which is a high-density DNA chip for food and animal feed testing. This chip is able to identify 15 fish, 12 mammals, and 5 bird species. However, the price of the equipment needed to perform the analysis is still too high to spread their use.

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Chapter 9

Authenticity of Tea (*C. sinensis*) and Tea Products

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This paper deals with the following aspects of tea authentication: (I) the differentiation of green and black tea can be achieved using ratios of different polyphenolic compounds, e.g. catechins vs. total phenolics or catechins vs. theaflavins. The concept of the ratio of total phenolics to total catechins is currently established by accumulation of more data. (II) To detect the geographic origin the flavonol glycosides ratios seem to be promising, but there is still a lack of data. (III) White teas are increasingly sold but there are not many data available on the constituents. It is currently impossible to assure the authenticity of white teas, especially of instant products. The ratio total polyphenols vs catechins is similar to green teas. (IV) The use and the amount of (instant) tea in RTD beverages such as ice tea is not always easy to detect depending on the process used to produce a cold water soluble tea. The authenticity can be checked using the flavonol pattern, in case of a harsh treatment by flavone C glycosides. Theanine could possibly be used as a marker in tea products. Methods are available for the determination and for the separation of D- and L-theanine. However, not much is known about the fate of theanine during the extraction and drying process.

Introduction

Is the authenticity of tea a real problem? In case of leaf tea an adulteration of tea with other herbs can be easily detected e.g. by microscopy. It is certainly different if instant products are used as in the production of these products additives can be used which might give rise to changes of certain constituents, e.g. solubilisation of tea cream by NaOH destroys the catechins. Another issue is the decaffeination. If solvents like ethylacetate are used the tea is not only decaffeinated but also “depolyphenolated”, in other words the concentrations of catechins and theaflavins are drastically reduced.

What else do we have to deal with?

There are variables in the plant material, the area of growth, the plucking standards, the plucking season and the technology (withering, drying, rolling among others). The determination of the geographic origin is in some cases of importance, e.g. claims have been made that there is more Darjeeling tea sold than produced. The differentiation of green and black teas by analytical means is not quite simple. White teas are increasingly sold but there is not much data available on endogenous constituents – the same is true for pu-erh teas.

The use and the amount of (instant) tea in RTD beverages such as ice tea is not always easy to detect depending on the process used to produce a cold water soluble tea. Processing like decaffeination also might change the flavonoid pattern depending on the process employed.

One of the most important problems for researchers outside the growing areas is to make sure that the samples used for authenticity work are really authentic.

Possible approaches

Possible concepts for the authentication of tea are the following:

Use of stable isotope ratios of a specific compound to detect geographic origin. An approach like that has been used for green coffees (1). In this work the $\delta^{13}\text{C}$, δ^{\square} and $\delta^{18}\text{O}$ values of caffeine have been determined by IRMS (isotope ratio mass spectrometry) and the data have been evaluated using linear discriminant analysis (LDA) and classification and regression trees (CART). The results were promising in case of the δ^{\square} and $\delta^{18}\text{O}$ values. This could also be a concept for tea. Another possible measure could be the mineral content or the aroma profile (see below). Polyphenols could also serve as authenticity markers, especially if the target compound is only present in a specific type of sample. Another useful concept is the ratio of specific individual compounds or groups of compounds.

Data available

Data for polyphenols in tea are available in the literature. There are lots of data for catechins e. g. (2-4). The situation is different for other groups of (poly)phenols. There are some data on flavonol glycosides (5-7), much less on flavone C glycosides (7, 8) and chlorogenic acids (7, 9) and nearly no data for proanthocyanidins (7, 10). Data obtained for the flavonols after hydrolysis and cleavage of the sugar moieties can be found in the literature but those are of limited value with respect to authentication. As regards black teas data on theaflavins are still scarce (11). Another problem is the quality of the data which are often affected by the methodology used. For total phenolics there is an ISO method available based on the Folin-Ciocalteu Assay (12). The same is true for the major catechins (13). For the determination of other polyphenols and flavonoids no standard methods are available. A special problem is the so called thearubigin (TR) fraction. No individual TRs have been really elucidated. The quantification (better estimation) of TR can only be achieved by calculation (14).

Green and black teas

The differentiation of green and black teas seems to be an easy task – at least for tea tasters. Depending on the plucking technique and other technological steps green tea should not contain theaflavins or thearubigin. The ISO working group on tea has set up a different concept: the differentiation by the catechin:total phenolics ratio. A ratio higher than 0.55 (0.5) is true for green teas and lower than 0.55 (0.5) for black teas. No figures for oolongs, white teas etc. have been discussed. Problems occur with special black teas, like Darjeelings or some Sri Lankan highland teas which have high amounts of catechins and low amounts of theaflavins. At the other end some low quality green teas are also very close to the breakpoint. It is necessary to employ validated methods to obtain the ratios. A common problem are the standards for calibration purposes. Sometimes the standards used are not pure and often the moisture content of e.g. the catechins or the theaflavins are unknown which give rise to a certain degree of variation of the data. This has been overcome in the ISO method by using a calibration against caffeine and relative response factors which have been determined in an international ring test with very well characterized catechin standards (13). The ISO standard method was published in 2005, however, the draft method has been employed for data collection purposes before that date. But there are other data generated with different methods, especially with calibration standards of unknown purity.

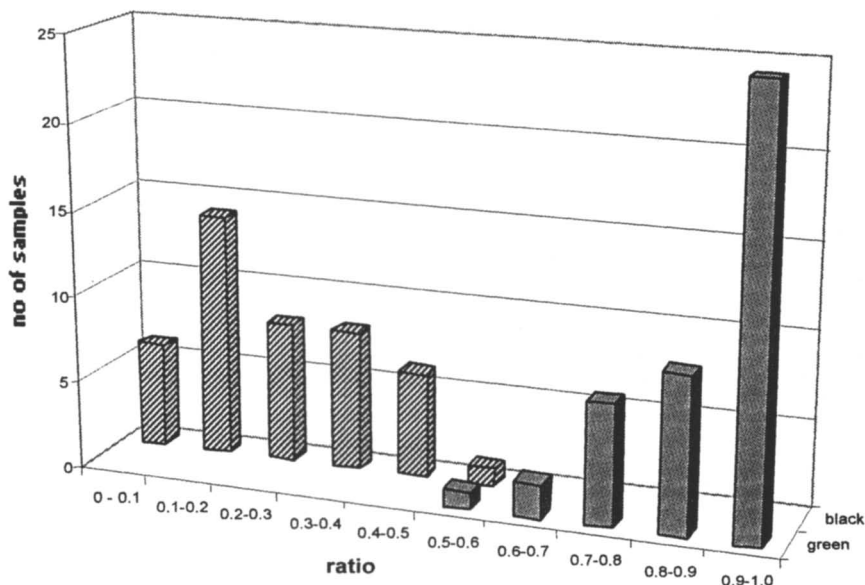


Figure 1: Ratio of total catechins vs total phenolics in green and black teas from the German market. The ratio is given in %.

Currently a data base is set up by the ISO working group to overcome these problems. The methodology agreed is the aforementioned Folin-Ciocalteu Assay for total phenolics and the HPLC determination of major catechins. Figure 1 shows this ratio for teas from the German market (11).

Other possible ratios are bisflavanols vs sum of bisflavanols and proanthocyanidins. The ratio was for green tea < 0.4 and for black tea > 0.4 . The concept proved to be valid for a limited set of samples (7). However, the determination of the proanthocyanidins and bisflavanols is complicated and the standards for calibration are not commercially available.

Another possible ratio is the one between the major catechins and the major theaflavins (11). A set of around 100 samples (green and black) has been analyzed and led to the conclusion that a ratio of 30 (catechin vs. theaflavins) was maximum for black teas. In the same work (11) another criterion was suggested: the content of galloylated catechin (for black teas $< 8\%$, for green teas $> 8\%$). Both concepts have the drawback that they are based on a limited number of samples.

Geographic origin

There are some papers in the literature regarding that issue. NMR profiling has been tested in connection with PCA and cluster analysis (15). The authors stated that the present results did not allow allocation of samples to individual countries (15), however, this approach has some potential.

Minerals have also been tested as with other foods as an authenticity marker (16). Fernández-Cáceres et al. (16) determined Zn, Mn, Fe, Mg, Cu, Ti, Al, Sr, Ca, Ba, Na and K by ICP-AES in 46 samples and applied PCA (principal component analysis), LDA and ANN (artificial neural networks) for the data treatment. From LDA calculations discriminant functions were obtained which gave a quite good separation of the different origins. This approach has to be validated with an extended set of samples (16).

Concepts based on isotope ratio determinations have not really been employed. The same is true for volatile compounds. There are some papers in the literature, e.g. a comparison of the volatiles from two different origins but this has not been tested with more samples (17). With regard to phenolic compounds there is currently no compound agreed which is specific for the origin. According to (7,18) there was one flavonol glycoside which only occurred in Chinese green teas but this finding is based on very few teas.

Winterstein and Finger (19) used flavonol glycosides as a tool to detect the geographic origin with special attention to Darjeeling samples. They employed multivariate statistics and model which showed a good correlation. The data set was limited (45 samples). In an earlier work Finger (20) stated that flavonol glycosides are potential substrates for polyphenoloxidases and some of the myricetine glycosides are partially degraded during the processing.

White teas and Pu-erh teas

There is not much information available in the literature on the composition of white teas. The results of the analysis of some white teas indicate that the caffeine content of white teas is relatively high (4.6 % for a set of 8 origin white teas) which is at the upper end of data for teas (21). The ratio between sum of the catechins and total phenolics is in the area of green teas (average ratio for the samples mentioned: 0.65), see Figure 2. The content of theogallin (3-galloylquinic acid) is also very high (average 1.73 %). Data on other polyphenols are not available as yet. There is no method available to detect the authenticity of instant white teas.

In case of pu-erh teas the situation is slightly better. Due to the production process the flavanol content is very low. According to Duh et al. (22) a pu-erh sample contained no flavanols except epicatechin. Kuo et al. (23) found flavanols (around 0.8 % as the sum of the main catechins) which is very similar to our own data (21). Data of Lin et al. (24) for three pu-erh teas were also very low with one exception (23 mg/g, extraction using 70 % ethanol). Again, not many data are available and there is no real concept to check the authenticity as yet.

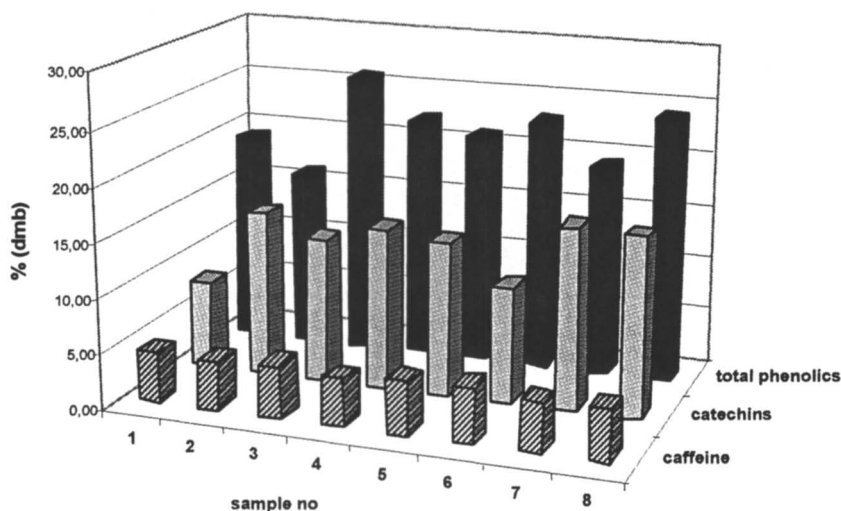


Figure 2: Catechin, caffeine and total phenolics in authentic white teas (21).

RTD beverages

After a boom for several years the market for RTD beverages such as ice tea is now stable on a high level. In former years in most countries drinks were based on freshly brewed or instant black teas. Currently also drinks based on green tea or beverages including also herbs or fruits are sold.

One of the concepts included the determination of caffeine and sometimes also theobromine. The presence of caffeine and/or theobromine does not necessarily mean that tea has been used and consequently this method is not useful as

authenticity marker. Other compounds among those the major flavonoids might be useful. If certain treatments in case of black teas for cream solubilisation are employed some characteristic compounds like flavanols or theaflavins are degraded and/or polymerized. Flavonol glycosides (FOG) do withstand most of the processes employed and the presence of the typical FOG can be used as a marker for tea. Moreover, as the concentration of flavonol glycosides is usually between 1-2 % on a leaf base it could be used as a semiquantitative estimation of the amount of tea used in the beverage. However, there are RTD beverages on the market which do not contain flavonol glycosides, probably due to the treatment during cream solubilisation. This leads to the suspicion of adulteration. It has to be mentioned that the fruit juices used in the RTD beverages may contain flavonol glycosides. In some cases this could be overcome by a determination of the tea-specific flavone C glycosides which are really stable compounds (7). According to (7, 8) the concentration range of the flavone C glycosides is 1-2 g/kg on a leaf base. Consequently the amounts to be detected in the RTD beverage are very low. The analysis is relatively complicated and far from being widespread agreed.

Theanine as authenticity marker

Theanine (γ -glutamylethylamide) is a non-proteinogenic amino acid which has been found nearly exclusively in *C. sinensis*. In the past few years it has entered the nutraceutical and also pharmaceutical sector as it has been claimed to have several beneficial effects (25, 26). Data from the literature concerning the theanine content of tea are between 0.1 and 3.4 % dmb. Own data using a newly developed simple HPLC method gave a wide range of 0.1 – 2 % dmb (27). Theanine is a chiral molecule. The method mentioned beforehand is not capable to separate D- and L-theanine, however, there are published methods for the separation of the enantiomers (28). Moreover, L- and D-theanine seem to have different physiological effects, at least in animal experiments (25). Theanine could be a good marker for the use of tea but there are some drawbacks, e.g. theanine can be synthesized either by chemical or biotechnological processes. The fate of theanine during the production of instant tea powders or RTD beverages is not well known. To summarize, theanine is currently not really a useful authenticity marker.

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.

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Chapter 10

Fruit Juice Authentication: What Have We Learned?

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The economic value and high-volume production of fruit juices have made them a likely target for adulteration with less expensive sweeteners and other ingredients. Over the past 30 years, chemists, trade associations and regulatory agencies have worked together to develop faster and more sensitive analytical methods for detecting fraud, along with a more complete compositional database for many commodities. Selected cases will be discussed that illustrate the advances that have been made in analytical chemistry. These include stable isotopic carbon analyses, SNIF-NMR, separation of *d* and *l* malic acid by chiral chromatography, analysis of trace oligosaccharides by GLC of TMS derivatives, and analysis of anthocyanin pigments with HPLC and electrospray mass spectroscopy (ESMS). Harsh penalties imposed by regulatory agencies and adverse consumer reactions to reports in the media have helped to reduce the severity of the problem.

Introduction

Fruit juice adulteration is regarded as a serious issue in the USA, whether one is a primary processor of fruit juice concentrates, a purchaser of fruit juice concentrates for use as a beverage ingredient, a regulatory official for FDA, a consumer with a strong advocacy for the health benefits of fruit juice, an analytical laboratory with clients in the beverage industry, a journalist with an agenda for uncovering consumer fraud, or an opportunist out to make a fast buck. It is instructive to examine selected cases of fruit juice fraud that have occurred in the USA over the past 30 years. Some received widespread media attention, while others were reported in professional journals and not mentioned in the popular press. All of these cases have helped to decrease the severity of the problem and all have had important economic consequences. The development of new and improved analytical methods for determining authenticity has been a major factor in reducing the problem. Significant contributions have come from commercial analytical laboratories as well as universities and government research laboratories. While FDA as the primary regulatory agency for food in the USA has major responsibility for combating fraud, the efforts of trade associations and individual companies have been very significant. Not to be overlooked is the role of the media. The impact of negative publicity on reputation and market share may be a greater deterrent than the fines levied by regulatory action.

Table I. Wholesale Prices of Fruit Juice Concentrates, \$/gal^a

Black Raspberry	143	Sour Cherry	33
Blueberry	79	Black Currant	27
Marion Blackberry	77	Concord Grape	10
Red Raspberry	72	Plum	9
Evergreen Blackberry	67	Dark Sweet Cherry	9
Pomegranate	45	White Grape	8
Strawberry	45	Apple	7
Red Currant	37	Pear	7
Cranberry	35		

^a Price estimates from an informal 2004 telephone survey of fruit juice concentrate producers.

Table I lists 2004 wholesale prices for fruit juice concentrates that were obtained from an informal telephone survey of fruit juice processors. The cost and availability of raw materials along with juice processing yield are major

factors influencing these prices, which are subject to sharp fluctuations from year to year. High-fructose corn syrup has a wholesale price of approximately \$2-3 per gallon. Hence it's obvious that money can be made through undeclared addition of inexpensive sweeteners. The extreme range in prices also reveals the potential problem of adding a less expensive fruit juice to a more expensive juice.

The Case of the Heavyweight Carbon Atom

Plants that use the Calvin Cycle for CO₂ fixation can readily be distinguished from Hatch-Slack plants by high-resolution MS of the CO₂ produced from their combustion. All plants discriminate in favor of the lighter carbon isotope, but Calvin cycle plants discriminate more. The proportions of ¹³C and ¹²C are compared with a reference calcium carbonate PeeDee Belemnite (PDB). The following formula is used to express the comparison to the standard:

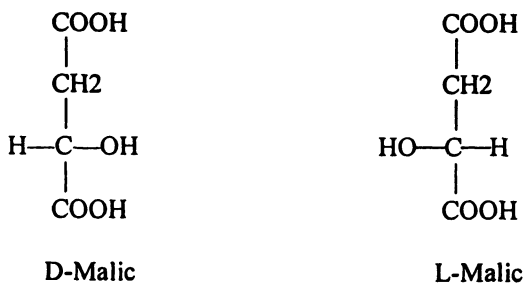
$$\delta^{13}\text{C} = \left[\left(\frac{^{13}\text{C}/^{12}\text{C}}{\text{sample}} \right) / \left(\frac{^{13}\text{C}/^{12}\text{C}}{\text{standard}} \right) - 1 \right] \times 10^3$$

Most fruits, including citrus, are Calvin Cycle and have $\delta^{13}\text{C}$ values ranging from -22 to -30 while Hatch-Slack plants range from about -9 to -20. Cane sugar and corn are Hatch-Slack. Several investigators demonstrated the effectiveness of stable isotopic carbon analyses for detecting corn syrup and cane sugar addition to orange juice, apple juice, honey, and maple syrup in the late 70's and early 80's. Both Landis Doner at the USDA laboratories in Philadelphia and Dana Krueger of Krueger Laboratories, a private commercial laboratory, were key contributors and have written excellent reviews (1,2,3). The problem was brought to the attention of many consumers with reports in the Boston Herald and the New York Times. In this instance, samples of apple juice were purchased from supermarket shelves by investigative journalists and sent to laboratories for analysis. Trade associations such as the Processed Apples Institute became pro-active, and initiated self-policing programs where products were monitored for authenticity. All of these actions— methods development, adverse publicity, self-monitoring programs, did much to reduce the practice of adulterating with corn syrup and cane sugar.

The Case of L vs. DL-Malic Acid

A landmark case in food law involves the adulterated apple juice for babies' scandal, which surfaced in 1988. Beech-Nut purchased apple juice concentrate

at a 20% cost advantage from Food Complex Inc. and Universal Juice Co. The concentrate contained little or no apple juice, the ingredients being invert beet sugar, corn syrup, malic acid, caramel coloring and imitation apple juice flavoring. Key analytical information which established fraud included detection of D-L (synthetic) malic acid using a combination of enzymic and HPLC analyses. Plants synthesize L-malic acid and not the D-isomer. Malic acid content of the juice as determined by HPLC was twice the value for L-malic determined spectrophotometrically using enzyme kits. The sensitivity for detecting synthetic malic acid has been improved with the ability to separate the isomers by chiral liquid chromatography (4). Figure 1 shows the separation of D-malic from L-malic acid. The LC method for determination of D-malic acid in apple juice has been adopted first action by AOAC International (5). The sugar profile also provided useful indices from both sorbitol content and the glucose:fructose ratio. The Beech-Nut apple juice contained no sorbitol and had an invert sugar pattern. Wrolstad and Shallenberger (6) had published a compilation from the literature of the free sugar and sorbitol content of fruits, and showed that apples accumulate more fructose than glucose and have a sorbitol content of approximately 5% of total sugars. Mattick and Moyer (7) did an extensive study of the composition of authentic apple juice; the three-year study encompassed 93 authentic varietal pure apple juice samples from major growing regions throughout the USA. Figure 2 compares the apple sugar profiles from the literature compilation with the data of Mattick and Moyer (8), the agreement being extremely close.



The Processed Apples Institute collected the evidence and prepared the case against Food Complex and International Juice, which was settled out of court. FDA took action against Beech-Nut and heavy penalties were given. More the \$2 million in fines was paid by Beech-Nut and company executives were both fined and given prison sentences. Losses in sales to Beech-Nut were estimated to be \$25 million. The case got widespread publicity in Business Week (9), Consumer Reports (10), the New York Times (11) and on Televisions "20/20"

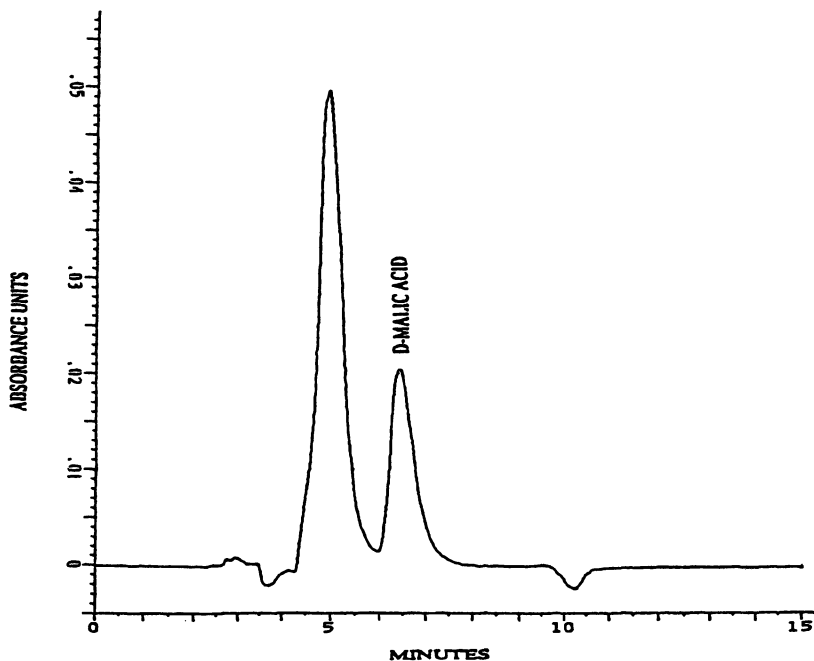


Figure 1. Chiral liquid chromatogram of apple juice containing D-malic acid; the large preceding peak is a combination of L-malic acid, citric acid, and other compounds found in apple juice that would form a complex with Cu II and L-valine. (Reproduced with permission from Reference 5. Copyright 1996.)

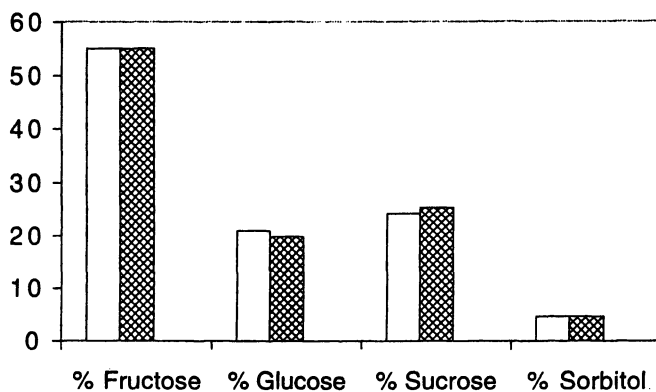


Figure 2. Bar chart comparing the % fructose, glucose, sucrose, and sorbitol (dry weight basis) as tabulated from a 14-sample literature compilation (Reference 6, open bars) and as reported in a 93-sample study (Reference 7, hatched bars). Figure is modified from figure 2, Reference 8).

program (12,13). Gerber Products Co., a competitor of Beech-Nut, unjustly suffered losses in sales from consumer reaction to the scandal. This case established that FDA takes adulteration seriously, even though it may not be a health hazard. It also documents the severity of possible economic consequences.

The Case for SNIF-NMR

It was clear from the Beech-Nut case that formulators were using invert beet sugar rather than high-fructose corn syrup or invert cane sugar as an undeclared sweetener in order to circumvent detection by stable carbon isotope analyses. This pointed to a need for analytical methods to detect the presence of invert beet sugar. G.J. Martin and colleagues at the University of Nantes and EUROFINS Laboratories developed a sophisticated method that can discriminate sugar origin on the basis of site-specific natural isotope fractionation (SNIF-NMR) (14,15). The method is based on the fact that the deuterium content at specific positions of the sugar molecules is different. A sugar sample is fermented to ethanol; the alcohol is distilled with a quantitative yield and analyzed with a high-field NMR spectrometer. The amounts of hydrogen and deuterium on carbons 1 and 2 are determined. A substantial authentic database needs to be developed for each commodity

CH₃CH₂OH vs. CH₂DCH₁DOD

with subsequent application of hierarchical clustering, principal component and discriminate analyses. Successful collaborative studies have shown that the methodology can be used to detect added beet sugar to concentrated and single-strength fruit juices (16) and to detect addition of either beet or cane sugar to maple syrup (17). Adulteration of maple syrup in North America has been uncovered by this methodology. Reg Muir of Mapleville USA worked with EUROFINS in establishing a database for authentic maple syrup and collecting the samples. The exposé was featured on ABC TV's "20/20" as well as in Vermont newspapers. The U.S. Attorney General's Office in Vermont prosecuted and convicted two criminal cases (U.S. District Court Criminal File No. 96-994 United States v. Roger Ames) (18). Recently, workers at Pennsylvania State University have used Fourier transform infrared (FTIR) spectroscopy and near-infrared (NIR) spectroscopy to detect beet and cane sugar addition to maple syrup. (19)

The Case of Hydrolyzed Inulin Syrup

Nicholas Low of the University of Saskatchewan has used trace oligosaccharide analyses to detect addition of sweeteners in fruit juices, honey and maple syrup (20). Isomaltose and maltose are marker compounds for high-fructose corn syrup, and reversion sugars (fructose-substituted sucrose) are marker compounds for invert beet and invert cane sugar. Using high-performance anion exchange liquid chromatography with pulsed amperometric detection (HPAE-PAD) he could detect the presence of invert beet sugar in orange juice at the 5% level, and 10% addition of high-fructose corn syrup to honey and maple syrup (20). Subsequently he used capillary gas chromatography of tri-methylsilyl derivatives with flame ionization detection (CGC-FID) for oligosaccharide fingerprinting (20). This methodology was faster and utilized less expensive instrumentation that was more readily available to analytical laboratories. Oligosaccharide fingerprinting by CGC-FID was successfully used to detect invert sugar and high-fructose corn syrup in orange (21,22) and pineapple juices (23). Figure 3 compares the CGC-FID chromatograms of pure apple juice with apple juice containing 10% high-fructose corn syrup.

Nicolas Low, working with David Hammond of the University of Reading, applied this methodology in a survey of commercial fruit juices. They detected "new" oligosaccharide peaks in several samples of apple juice, which they had not observed before in apple juice, or in common sweeteners. Subsequently they identified the added material to be a new sweetener, high-fructose syrup derived from hydrolyzed inulin (source Jerusalem artichoke or chicory root). Figure 4 compares the CGC-FID chromatograms of pure apple juice with apple juice containing 10% hydrolyzed inulin syrup. The adulteration was traced to two companies, one located in Belgium and one in Holland. The contaminated apple juice concentrate was purchased by several USA juice processors, and found its way into several major fruit juice brands. The incident received widespread publicity, and was featured on ABCs "20/20". There was increased surveillance by FDA, withdrawal of large amounts of product from supermarket shelves, and several cases of litigation. The method was successfully peer-validated for identification of hydrolyzed inulin syrup and high-fructose corn syrup in apple juice (24)

The Case of Immigrant Acids and Alien Anthocyanins in Cranberry Juice

Our laboratory was extensively involved in a case concerning adulterated cranberry juice cocktail (25,26). Minot Food Packers Inc., a processor of cranberry products and co-packer of a number of juice products in Bridgeton,

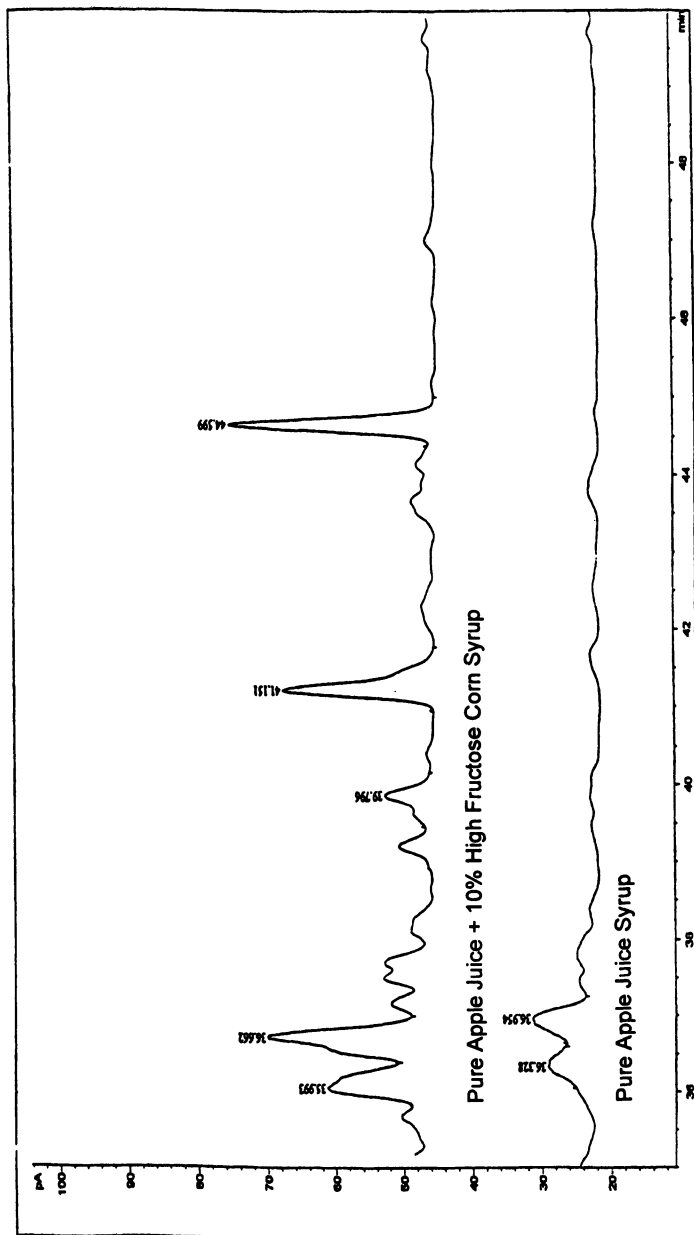


Figure 3. Capillary gas chromatogram of trimethylsilyl (TMS) derivatives with flame-ionization detection (CGC-FID) of pure apple juice and apple juice containing 10% high-fructose corn syrup. (Reproduced with permission from Reference 20. Copyright 1998 Blackie Academic and Professional.)

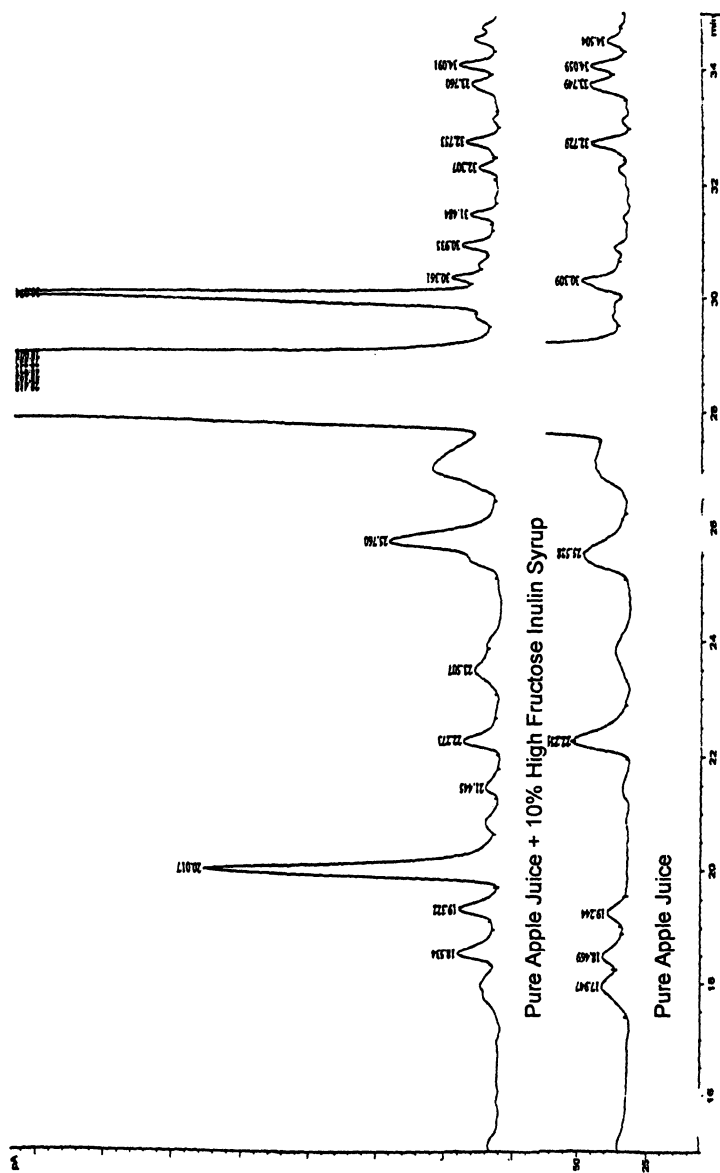


Figure 4. Capillary gas chromatogram of trimethylsilyl (TMS) derivatives with flame-ionization detection (CGC-FID) of pure apple juice and apple juice containing 10% hydrolyzed inulin syrup. (Reproduced with permission from Reference 20. Copyright 1998 Blackie Academic and Professional.)

N.J. suspected that some of their competitors were manufacturing a product that did not meet commercial specifications. Their suspicions were based on sensory qualities including taste, astringency and visual appearance along with economic factors (fruit and ingredient costs vs. sales price.) They could not verify their suspicions in the laboratory, however. Discussions in our laboratory led to their funding a research project for development of analytical methods and a compositional database for authentic cranberry juice. Authentic fruit was obtained from Minot Food Packers and Ocean Spray Cranberries, Inc. The cranberries were processed into juice in our pilot plant using a process typical of commercial practice.

The following compositional measurements were made on the juices: Titratable acidity, °brix, specific gravity, uv-visible spectral scans, total monomeric anthocyanins, polymeric color, Hunter CIEL*a*b* indices, stable isotope carbon ratios, sugars, nonvolatile acids, and anthocyanidins. Sugars were determined by HPLC using an anion-exchange carbohydrate column and refractive index detection. Cranberries are unusual in that they accumulate more glucose than fructose, having a glucose: fructose ratio of 3.8. This index as well as stable carbon isotope analyses would not be useful in the analysis of cranberry juice cocktail, however, since high-fructose corn syrup is a major ingredient. Quinic, malic, citric and shikimic acids were separated and quantitated by HPLC using UV detection. This assay proved to be very useful as cranberries have a distinctive and consistent profile with a substantial amount of quinic acid. Quinic acid content, in fact, is used industrially as an index for cranberry juice content (27, 28). We elected to analyze cranberry anthocyanidins rather than the naturally occurring anthocyanin glycosides. Sample preparation includes isolation of the anthocyanins by solid-phase extraction, hydrolysis with 2N HCl at 100°C, followed by isolation of the anthocyanidins with solid-phase extraction (25). This process is time-consuming, and the anthocyanidins are very unstable. Our reason for analyzing anthocyanidins rather than the anthocyanin glycosides was the limitation of LC columns at that time. Endcapped C-18 columns were not yet available, and we obtained very poor resolution in our attempts to separate complex anthocyanin mixtures on C-18 columns. While over 600 anthocyanin pigments have been identified in nature (29), there are only six common anthocyanidins (Figure 5), which we could separate by HPLC. Figure 6 shows the chromatogram of the anthocyanidins in authentic cranberry juice. The two peaks (cyanidin and peonidin) are broad and show pronounced tailing.

We were successful in detecting adulteration in preliminary tests on samples supplied to us by Minot, and we subsequently analyzed blind a set of 32 commercial samples. We concluded that 19 of the samples were adulterated (26). Addition of both citric and malic acids was detected by HPLC, and the presence of grape-skin extract was detected by analysis of the anthocyanidins. Figure 7 shows the anthocyanidin chromatogram of an adulterated commercial

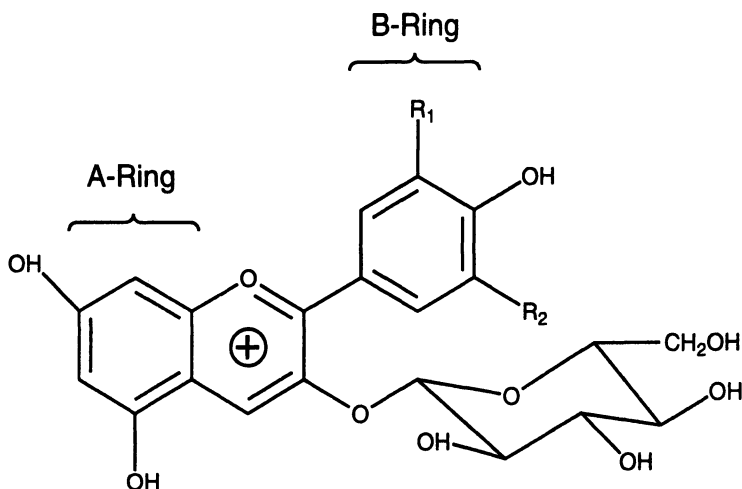


Figure 5. Generalized structure for anthocyanin pigments. Pelargonidin, R_1 & $R_2 = H$; cyanidin, $R_1 = OH$, $R_2 = H$; delphinidin, R_1 & $R_2 = OH$; peonidin, $R_1 = OMe$ & $R_2 = H$; petunidin, $R_1 = OMe$ & $R_2 = OH$; malvidin, R_1 & $R_2 = OMe$.

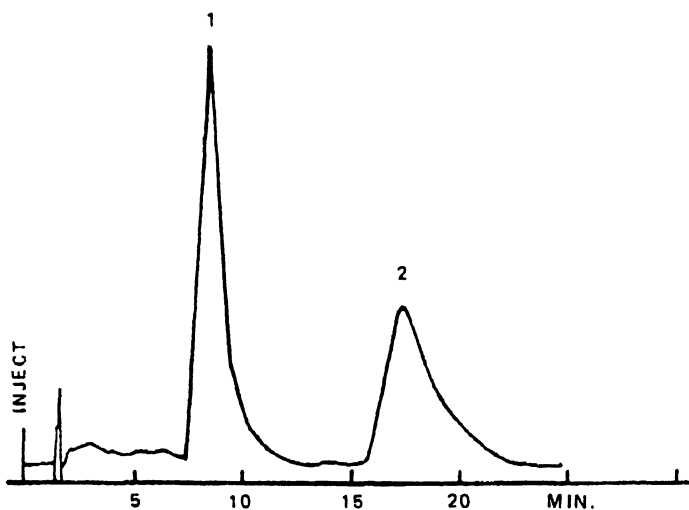


Figure 6. HPLC chromatogram of cranberry anthocyanidins, Peak 1 = cyanidin; Peak 2 = Peonidin. (Reproduced with permission from reference 25. Copyright 1986.)

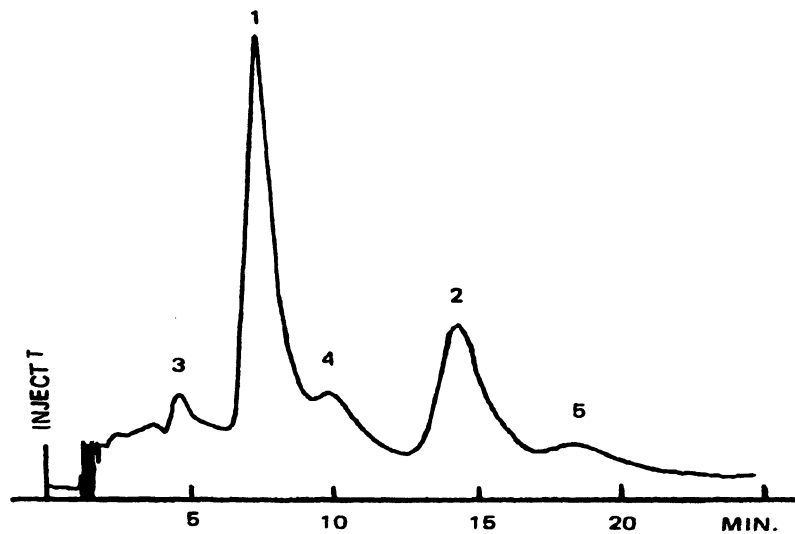


Figure 7. HPLC chromatogram of anthocyanidins in an adulterated commercial cranberry juice drink. (Reproduced with permission from reference 26. Copyright 1986.)

cranberry juice drink. Comparison with the simple chromatogram of authentic cranberry anthocyanidins (Figure 6) reveals presence of delphinidin, petunidin, and malvidin in addition to cyanidin and peonidin. Grape-skin extract is the likely source for the additional pigments. The estimated cranberry juice content for the adulterated samples, which were being sold as 20-25% juice, was typically 15%, and as low as 3%. When presented with the evidence, the guilty parties admitted to their misdeeds and an out-of-court settlement was reached. We presented our results at the 1984 meeting of the AOAC and were concerned when Food Chemical News reported that Oregon State University researchers had found the "majority of cranberry juice drink samples to be adulterated" (30). This statement gave a false impression of the magnitude of the problem in the commercial marketplace. The sampling had been conducted to track suspect small to medium-sized firms and was not representative of the market. While the sample set included cranberry juice cocktail produced by Ocean Spray Inc., these were tested as authentic. Because of Ocean Spray's dominance of the cranberry juice cocktail market, they would very likely suffer a loss in sales with any negative publicity on adulterated cranberry juice. A very real dilemma is that those firms that produce a legitimate product and take action to stop adulteration may very well suffer a financial loss because of adverse publicity. Elia Coppola and co-workers at Ocean Spray, Inc. have published extensive cranberry compositional data and continued to improve the methodology for determining authenticity (27,28). These efforts illustrate how commercial firms can work to combat the problem and protect their industry's integrity.

Progress in Anthocyanin Pigment Analyses

Pigment resolution was greatly improved with the availability of en-capped columns. Figure 8 shows the HPLC separation of anthocyanidins, and Figure 9 showing a typical separation of the anthocyanins in authentic cranberry juice. HPLC analysis of complex matrices containing 15 and more anthocyanins is now routine. Use of anthocyanin pigment analyses will be of no use for testing the authenticity of orange, apple and pineapple juice, the major fruit juices of commerce, since they contain no anthocyanins. Referral to Table 1, however, shows that those juices of highest economic value are also high in anthocyanin pigment content. Juices of high economic value will be likely targets for adulteration. Addition of sweeteners and/or low-cost juices will dilute the pigment concentration, with the resulting temptation to add anthocyanin-containing colorants or juices to meet purchase color specifications. Anthocyanin matrices have distinctive profiles, and many contain marker compounds that can now be identified by LC-MS and MS-MS, even when present as minor peaks (29). For example, cyanidin-3-sophoroside-5-glucoside acylated with sinapic acid can serve as a marker for red cabbage extract and

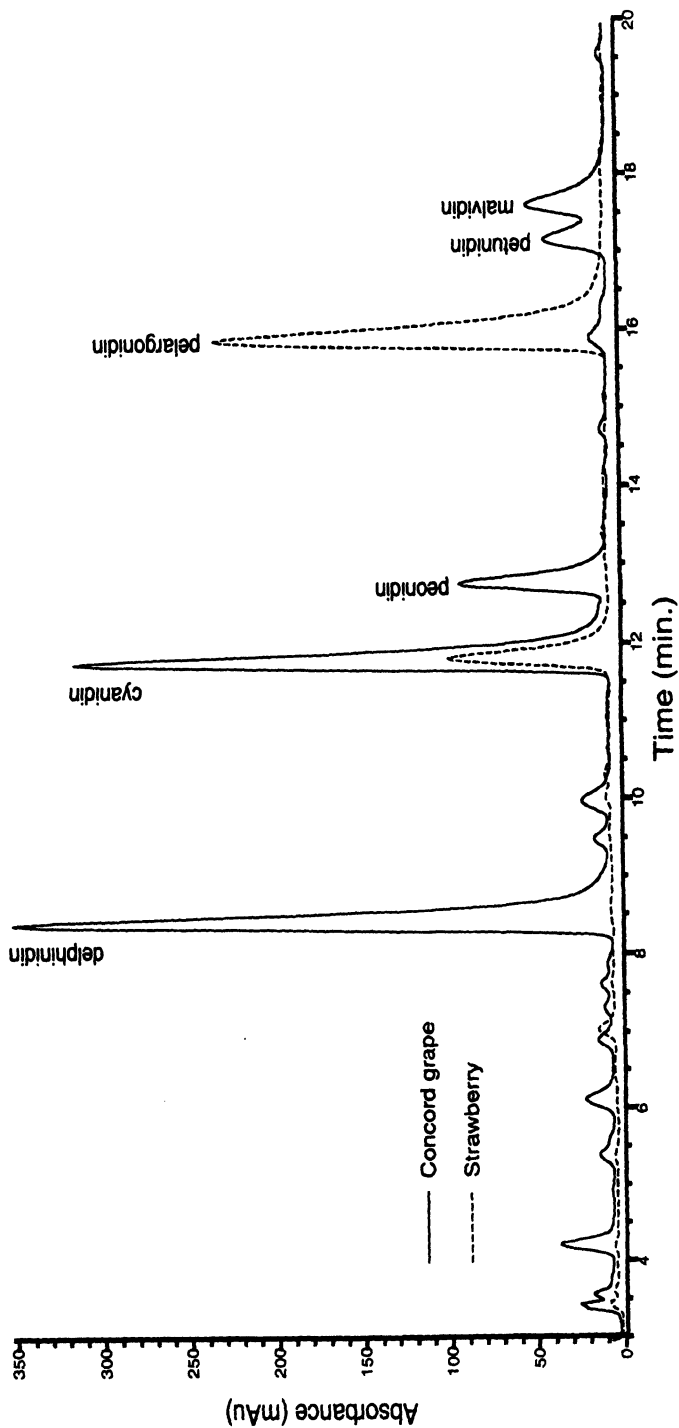


Figure 8. HPLC chromatogram of anthocyanidins. Sources for delphinidin, cyanidin, petunidin, peonidin, and malvidin are Concord grape juice, and the source for pelargonidin is strawberry juice.

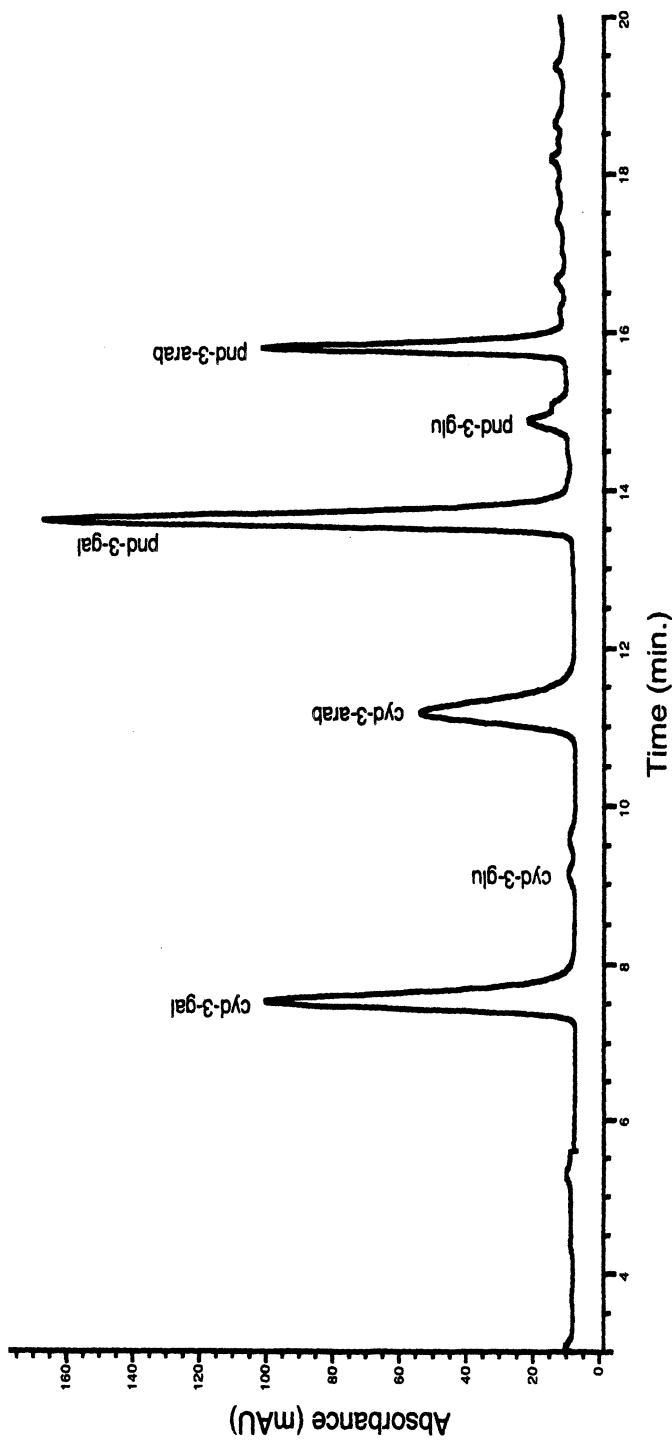


Figure 9. HPLC chromatogram of cranberry anthocyanins. Elution order. Cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside, peonidin-3-galactoside, peonidin-3-glucoside, peonidin-3-arabinoside.

cyanidin-3-xylosylglucosylgalactoside acylated with sinapic acid can be a marker for black carrot extract (31). We have detected adulteration of commercial strawberry juice concentrate with black currant from the presence of the 3-glucosides and 3-rutinosides of cyanidin and delphinidin (unpublished data).

What have we learned?

It is instructive to take a historical perspective in examining the issue of determining authenticity of foods. In these cases involving fruit juices, it is clear that improved analytical methods can play a major role in detecting fraud. In several cases, methods with increased sensitivity permitted detection of adulteration that had been below the detection level of older testing methods. In most cases of successful litigation, identification of the adulterant was accomplished through analytical chemistry. At a 1996 ACS Symposium, Sam Page of FDA's Center for Food Safety and Applied Nutrition stated, "Chemists are cheaper than lawyers" (32). He advised manufacturers to adopt state-of-the-art screening procedures and set tight contract specifications for suppliers. He advised, "It's easier to reject a shipment because it fails to meet specifications than to prove adulteration in a court of law". Advances in determining authenticity have been achieved through the cooperative efforts of industry, trade associations, academia, and regulatory agencies. While severe legal penalties are a deterrent, the prospects of adverse publicity from the media may be an even larger disincentive. We need to be vigilant, but also realize that we can't change human nature. If there is the possibility of making substantial profit with low risk of getting caught, someone is going to be willing to take the risk.

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Chapter 11

Wine Authentication Using Stable Isotope Ratio Analysis: Significance of Geographic Origin, Climate, and Viticultural Parameters

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Stable isotope ratio analysis of wine by ²H-SNIF[®]-NMR, ¹³C-, and ¹⁸O-isotope ratio mass spectrometry are official methods in the European Union for the proof of chaptalization, addition of water, sweetening with sugar, and authentication of geographic origin and year of harvest. By evaluation of stable isotope data of authentic reference wines from the German wine-growing regions Franconia and Lake Constance, Hungary, and Croatia as well as wines which were on the German market, the influence of geographical origin, climate, year and date of vintage, and viticultural aspects (soil water status, water stress, irrigation) on isotope fractionation of ¹³C, ²H, and ¹⁸O in water, sugar, and alcohol are discussed.

Review on authentication of wine by stable isotope ratios

Authentication of wine is the analytical process by which a wine is verified as in compliance with its label description. Adulteration of wine was already prosecuted and punished at the age of Hamurabi (1792 - 1750 B.C.), but also nowadays mislabelling of geographic origin, addition of sugar and/or water to pretend a better wine quality or to increase its amount are ascertained again and again. Since these adulterations became more and more sophisticated, it was necessary to develop sophisticated analytical methods for a significant proof of wine authenticity. During the last 30 years many studies on the intermolecular and intra-molecular non-statistical distribution of stable isotopes of the bio-elements ^{13}C , ^2H , ^{18}O , ^{15}N , and ^{34}S in natural compounds have been published; it was found that the distribution of stable isotopes in bio-molecules is controlled by logical principles including biotic and abiotic fractionation processes which result in a pattern characteristic for the plant species and its geographical origin (1). The most important biotic fractionation is that of carbon and hydrogen which takes place during photosynthesis and biosynthesis of sugar and other components in the plant. $^{13}\text{C}/^{12}\text{C}$ - and $^2\text{H}/^1\text{H}$ -isotope ratios of sugar and its related ethanol obtained by fermenting in the same water are primarily determined by two different biosynthetic pathways of biological carbohydrate formation, the Hatch-Slack pathway of C_4 -plants such as corn and sugar cane with higher ^{13}C - and ^2H -concentrations, and the Calvin pathway of C_3 -plants such as wheat, sugar beet, or grapevine. The knowledge of stable isotope fractionation in plants and their fruits promised to be a powerful tool for authentication of food products produced from these raw materials. Cases of adulteration of fruit juice or control of chaptalization of wines with sucrose which is restricted to some northern situated wine-growing regions of the EU, stimulated further research to develop reliable methods for the proof of such oenological treatments. Stable Isotope Ratio Analysis (SIRA) in official wine control started 1990 in the EU by adopting the 'SNIF[®]-NMR-Method' for the detection of chaptalization of grape must and wine by ^2H -Nuclear Magnetic Resonance (NMR) of ethanol (2), followed by ^{18}O - and ^{13}C -Isotope Ratio Mass Spectrometry (IRMS) (3,4). Table I summarizes the relevant methods, molecules, isotopomers, ratios, symbols, and units of SIRA. The principle of the SNIF[®]-NMR-method, developed by Prof. Martin (5,6,7) is based on the observation that the deuterium of the sugar- and water-molecule is transferred during fermentation by Site-Specific Natural Isotope Fractionation ('SNIF') into the methyl- and the methylene-position of the ethanol molecule. Approximately 85 % of deuterium in the sugar molecule are transferred during fermentation into the methyl-group of ethanol expressed by the $(\text{D}/\text{H})_1$ -ratio and about 75 % of the

deuterium of grape water into the methylene-group of ethanol expressed by the $(D/H)_{II}$ -ratio.

Table I. Stable isotope ratio analysis used for wine authentication

<i>Analytical Method</i>	<i>Component Isotopomer</i>	<i>Symbol, unit</i>	<i>Authentication parameters</i>
SNIF [®] - NMR	CH ₂ DCH ₂ OH CH ₃ CHDOH	(D/H) _I ; ppm (D/H) _{II} ; ppm	proof of chaptalization geographic origin
¹³ C/ ¹² C- IRMS	Ethanol, sugar, organic acids glycerol, CO ₂	δ ¹³ C; [‰] V-PDB	geographical origin, climate proof of C ₄ -sugar, synthetic glycerol, acids, and CO ₂
¹⁸ O/ ¹⁶ O - IRMS	Water, ethanol,	¹⁸ O/ ¹⁶ O δ ¹⁸ O; [‰] V-SMOW	geographical origin, year of vintage, addition of water

D= Deuterium (²H); V-SMOW= Vienna Standard Mean Ocean Water, δ¹⁸O= 0 ‰;
V-PDB= Vienna Pee Dee Belemnite, δ¹³C= 0 ‰ V-PDB

The $(D/H)_I$ -ratio represents the botanical origin of the fermented sugar whereas the $(D/H)_{II}$ -ratio is typical for the deuterium content of the grape-water and reflects the climatical conditions related to the geographical origin and the year of vintage. Figure 1 elucidates that the $(D/H)_I$ -ratio of ethanol from beet sugar (92.5 +/-1 ppm) is significantly lower compared to that of ethanol from wine (98-104 ppm, extreme cases up to 107 ppm). By chaptalization of grape must the original $(D/H)_I$ -ratio of the related ethanol significantly decreases in correlation with the amount of beet-sugar. In the same way the $(D/H)_I$ -ratio increases by use of C₄-sugars like cane sugar with $(D/H)_I$ -ratios between 109 and 112 ppm. Since it is possible to use mixtures of beet and cane sugar, in order to simulate wine-typical $(D/H)_I$ -values, it is always necessary to determine both the $(D/H)_I$ - and δ¹³C-value of grape sugar and wine ethanol respectively; the ¹³C-amount does not change significantly in case of an addition of beet sugar, but dramatically by use of cane sugar.

The significance of the ¹⁸O/¹⁶O-ratio of water in grapes and wine was already discussed in 1982 by Dunbar (8). Due to evaporation of water in the grape during ripening, an enrichment of ¹⁸O takes place, leading to values higher than those of the ground water which is transported via roots into the fruits. This process is influenced by geographic origin and climate. The δ¹⁸O-value in grape water therefore is a characteristic marker for the geographical origin of the grapevine and its climate, and can be used to prove addition of ground- or tap-water (8, 9, 10, 12, 13, 17, 18).

Since authenticity testing of a wine by SIRA must be related to analytical data of a set of reference samples which are as close as possible to origin, year, date of vintage, and cultivar of the sample to be analysed, it was necessary to establish an official databank which contains authentic and representative samples of all EU wine-growing regions. Corresponding to the actual regulation of the European Commission (11) for this analytical EU Wine Databank (EUWDB), about 1400 samples of grapes (15 kg) are taken each year by official controllers in the wine-growing regions of the EU; after micro-vinification the wines are analysed with SIRA in official institutes and the data enter the EUWDB. SIRA actually is not only used for the authentication of wine (7,9,12, 13), fruit juice (14), and spirits (15) but also for authentication of many other food products (16).

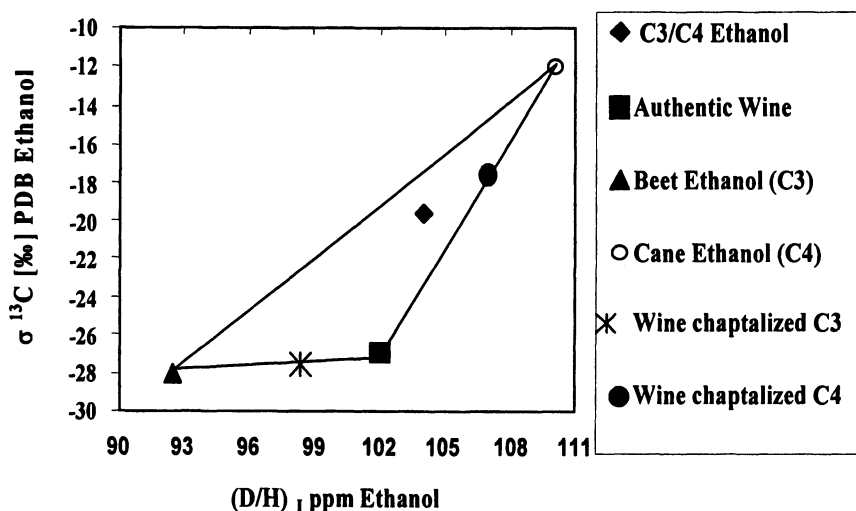


Figure 1. Proof of chaptalization or sweetening of wine with beet-, cane-sugar, and mixtures of beet- and cane-sugar by SNIF-NMR[®] and ¹³C-IRMS of ethanol.

Material and Methods

The determination of (D/H)- and ¹³C/¹²C-isotope ratios of ethanol in wine and of ¹⁸O/¹⁶O-isotope ratio in grape and wine water were performed according to the official analytical methods of the EU. For ²H-SNIF-NMR[®]-analysis (2)

authentic grape-musts were fermented with yeasts (*Saccharomyces Cerevisia*). Wines were distilled with spinning band columns using automatic vapour separation. Water content of distillates (6-9 % mas) was determined by densitometry. Acquisition of ^2H -NMR spectra of ethanol was performed by a BRUKER 400ARX spectrometer with a fluorine lock-channel and a probe-head tuned to the deuterium frequency of 61.42 MHz. 2.0 ml distillate and 2.0 ml TMU (N,N-Tetramethylurea, Reference Standard STA003, Institute for Reference Materials and Measurements, B-2440 Geel, Belgium) were weighed in a bottle and transferred into a 10 mm NMR-tube, adding 50 μl C_6F_6 as a lock substance. The deuterium spectra were recorded with an acquisition time of 6.7 s, a 25 μs pulse (90° flip angle) and 10 experiments per sample with 256 scans each. Processing of the spectra and calculation of $(\text{D}/\text{H})_{\text{I}}$ - and $(\text{D}/\text{H})_{\text{II}}$ -ratios versus the certified (D/H) -ratio of TMU was performed with EUROSPEC[®] software. The standard deviation of $(\text{D}/\text{H})_{\text{I}}$ -measurement is less than 0.4 ppm (2).

For $^{13}\text{C}/^{12}\text{C}$ -IRMS of ethanol a Vario EL elemental analyser (Elementar Analysensysteme GmbH, Hanau, Germany), equipped with a solid sample auto-sampler on top of the combustion furnace was used for fully automated stable isotope analysis. Ethanol samples (3 μl) and reference materials were filled into gas-tight tin capsules using a microliter-syringe and the capsules were sealed by a mechanic capsule press. The EA was on-line connected with a GVI2003 (GV Instruments Ltd. Manchester, UK) mass spectrometer, suitable for the measurement of stable isotope ratios of carbon, nitrogen, oxygen and sulphur. Control of the analyses and data evaluation was performed by a GVI software. Usually three samples each containing 3 μl of equivalent quantity of a distillate, are combusted and the carbon isotope ratio of the CO_2 formed is determined. The calibration of combustion and isotopic determination were performed using the international carbon isotope standard NBS-22 (NIST-22), for which a value of -29.8 ‰ has been accepted. The standard deviation of measurement was less than 0.1 ‰ for three measurements of the same sample (4).

$\delta^{18}\text{O}$ -values were determined with a Finnigan-MAT261 or Thermo-Finnigan DeltaXLplus-IRMS using the ions m/z 46 ($^{12}\text{C}^{16}\text{O}^{18}\text{O}$) and m/z 44 ($^{12}\text{C}^{16}\text{O}_2$) which are obtained after equilibrium of the isotope exchange of water and carbon dioxide. The exchange reaction $^{12}\text{C}^{16}\text{O}_2 + \text{H}_2^{18}\text{O} \leftrightarrow ^{12}\text{C}^{16}\text{O}^{18}\text{O} + \text{H}_2^{16}\text{O}$ proceeds via the solved hydrogen carbonate and is temperature dependant. The carbon dioxide in the vapour phase is used for analysis. For equilibration the 10 ml sample flasks were filled with 0.5 ml sample and 0.3 % carbon dioxide gas in helium is flushed with a stream of 100 ml/min at least 5 minutes (total gas volume should be about 500 ml). The batch of sample bottles was equilibrated in a temperature controlled room (22 °C). After equilibration the carbon dioxide of the sample bottles was transferred into the IRMS through a Nafion-membrane to remove water vapour and ethanol. The measurements were performed versus calibrated laboratory standard water. The relative difference of the ion intensity

ratio of m/z 46 and 44 (I46/I44) between the samples and standards were measured in ‰ and expressed in the relative difference $\delta^{18}\text{O}$ ‰ versus international standard V-SMOW (Vienna Standard Mean Ocean Water). The standard deviation of $\delta^{18}\text{O}$ -measurement was less than 0.15 ‰ (3).

Influence of year of vintage and climate on isotope ratios

The influence of year of vintage and climate on stable isotope ratios in wine can be discussed best by data of authentic samples from several years and the same wine-growing region. The Bavarian Health and Food Safety Authority (LGL) is responsible for the EUWDB and SIRA of authentic wines of the wine-growing regions Franconia and the Bavarian district of Lake Constance.

Table II. Mean stable isotope data of authentic wines from Franconia (n/year = 50), vintages 1995 to 2003; P= precipitation (Aug.-Oct.), S= sunshine (Aug./Sept.), and T= annual temperature for City of Würzburg

<i>Year</i>	<i>P</i> <i>mm</i> <i>L/m²</i>	<i>S</i> <i>h</i>	<i>T</i> <i>°C</i>	$\delta^{18}\text{O}$ ‰ <i>V-SMOW</i> <i>Water</i>	$(D/H)_I$ <i>ppm</i> <i>Ethanol</i>	$(D/H)_{II}$ <i>ppm</i> <i>Ethanol</i>	$\delta^{13}\text{C}$ ‰ <i>V-PDB</i> <i>Ethanol</i>
1995	217	327	15.9	-3.39	101.1	124.7	-29.4
1996	187	299	14.8	-3.06	101.3	124.7	-29.1
1997	70	500	18.1	0.44	100.9	124.3	-27.3
1998	290	347	16.4	-3.82	100.4	122.2	-28.1
1999	77	423	18.2	-0.59	100.7	125.0	-27.0
2000	125	393	17.0	-1.97	101.3	124.0	-28.5
2001	127	323	16.1	-2.40	100.4	124.7	-28.0
2002	120	398	16.5	-2.4	100.4	124.6	-28.0
2003	66	549	19.2	4.0	102.7	128.2	-25.7
<i>Annual mean</i>	<i>155</i>	<i>361</i>	<i>16.4</i>	<i>-1.3</i>	<i>101.2</i>	<i>124.8</i>	<i>-27.8</i>

SOURCE: Meteorological data from German Weather Service

The wine-growing region Franconia with 6000 ha (15000 acres) vineyards is located along the River Main in Northern Bavaria (latitude: 50.1 to 49.6°; longitude: 9 to 10°). The period of grape picking usually starts mid of September and ends late October with exception of the year 2003, where the main vintage already was finished end of September. The data in Table II show that the

variability of stable isotopes is mostly expressed by the oxygen isotope ratio of wine water but also by isotope ratios of hydrogen and carbon. The annual variability of the stable isotope fractionation reflects the variability of the meteorological conditions like precipitation, sunshine, and temperature during the ripening period from July until the date of grape picking. Observations that the $\delta^{18}\text{O}$ -value of wine water is significantly influenced by precipitation during the period before harvest (10,13,17,18,21) can be confirmed by meteorological data; the maximum precipitation of 290 mm between August and October was observed in 1998 (180 mm in October), leading to the lowest $\delta^{18}\text{O}$ -values (mean $\delta^{18}\text{O} = -3.8$ ‰) in the years under observation. In contrary the $\delta^{18}\text{O}$ -values of wines from 2003, a year with low precipitation in August/September (33 mm) and October (33 mm), high temperatures, and number of sunshine-hours, were elevated to a mean value of +4 ‰, which normally is typical for wines from South Italy (Figure 3). The significant effects of extremely different meteorological conditions in Franconia 1998 and 2003 as well as other years under observation are also reflected by the other stable isotope ratios (Table II); in 2003 also the highest $\delta^{13}\text{C}$ -, $(\text{D}/\text{H})_{\text{I}}$ -, and $(\text{D}/\text{H})_{\text{II}}$ -values of ethanol were determined.

Table III summarizes the stable isotope data and precipitation data for authentic wines from the small wine-growing region 'Bavarian Lake Constance' (latitude: 47.5°; longitude: 9.7°) which has only 22 ha (55 acres) of vineyards; the region is located about 300 km in the South of Franconia near the City of Lindau, close to the Alps.

Table III: Mean stable isotope data of authentic wines from Lake Constance (n/year = 10), precipitation P (Aug. – Oct.), vintage 1997-2003

<i>Year</i>	<i>P</i> <i>mm</i> <i>L/m²</i>	$\delta^{18}\text{O}$ <i>‰ V-SMOW</i> <i>wine water</i>	$(\text{D}/\text{H})_{\text{I}}$ <i>ppm</i> <i>ethanol</i>	$(\text{D}/\text{H})_{\text{II}}$ <i>ppm</i> <i>ethanol</i>	$\delta^{13}\text{C}$ <i>‰ V-PDB</i> <i>wine ethanol</i>
1997	160	-2.94	100.2	123.2	-29.4
1998	298	-5.56	99.5	120.5	-28.8
1999	183	-4.58	100.0	121.5	-29.5
2000	241	-3.60	101.1	124.5	-29.5
2001	246	-5.30	100.4	122.6	-29.6
2002	328	-5.60	99.5	122.2	-28.7
2003	186	-0.82	101.3	124.2	-29.1

SOURCE: meteorological data from German Weather Service registered August to October for City of Constance

During the years 1997 and 2003, always negative $\delta^{18}\text{O}$ -values (minimum -5.6 ‰) and rather low $\delta^{13}\text{C}$ -values (min. -29.6 ‰) were determined. The

stronger depletion in this region can be explained by significantly higher precipitation than in Franconia. The lower precipitation in 1997 and 2003 was leading to less negative $\delta^{18}\text{O}$ -values, however the $\delta^{13}\text{C}$ -values did not increase; apparently further parameters like air humidity, soil water capacity, or ground water level which are known to have an influence on stable isotope fractionation of carbon (10, 21, 18, 19) are responsible for the almost constant low $\delta^{13}\text{C}$ -values. Further studies are necessary to investigate and quantify these effects.

In case of authentication of a wine by the $\delta^{18}\text{O}$ -value of water it is not only necessary to know the climatic conditions during the ripening period but also the date of grape picking. Figure 2 shows by example of Franconian wines 2003, a decrease of the $\delta^{18}\text{O}$ -value from the beginning of the harvest in late August (maximum values in week no. 35: + 7 ‰ V-SMOW) to the end of October (minimum values in week no. 44: +1.5 ‰ V-SMOW); negative $\delta^{18}\text{O}$ -values were observed in wines of late harvested grapes in November or December (Ice Wine).

Also in other European wine growing regions, even in South Italy, this decrease of the $\delta^{18}\text{O}$ -value is more or less significant, depending on the meteorological conditions like precipitation and temperatures which influence the evaporation rate of grape water. The decrease of isotope ratios related to date of vintage can be observed also for $(\text{D}/\text{H})_{\text{II}}$ -values but not as significant for $\delta^{13}\text{C}$ - and $(\text{D}/\text{H})_{\text{I}}$ -ratios of ethanol (12, 13, 20).

Influence of geographic origin on stable isotope ratios

For authentication of wines from countries outside the EU, databanks with authentic reference samples are not available. Therefore in Germany a 'Third Countries Databank' was established with the aim to collect traditional analytical and stable isotope data of wines imported to the German market. During 1997 to 2001 it was even possible to analyse additional authentic wines from Hungary and Croatia by a co-operation of the LGL Würzburg with the National Hungarian Institute for Wine Qualification, Budapest and the Croatian Institute of Viticulture and Enology, Zagreb (21). Figure 3 shows by example of the year 2000 the correlation of mean $(\text{D}/\text{H})_{\text{II}}$ -values of ethanol and $\delta^{18}\text{O}$ -values of wine water of authentic wines from Hungary, Continental Croatia, and Coastal Croatia, as well as commercial wines from Bulgaria, Macedonia, Yugoslavia, and Romania taken from the German retail shops. In order to compare the correlation with data of wines from further European countries, also some German and Italian wine-growing regions are included. Figure 3 confirms that mean $\delta^{18}\text{O}$ - and $(\text{D}/\text{H})_{\text{II}}$ -values which both represent isotope fractionation of grape water, are well correlated ($r = 0.962$). In contrary to the exceptional dry and hot year 2003 (cf. Table II) German wines from 2000 had significantly lower isotope ratios than those from southern and south-eastern European

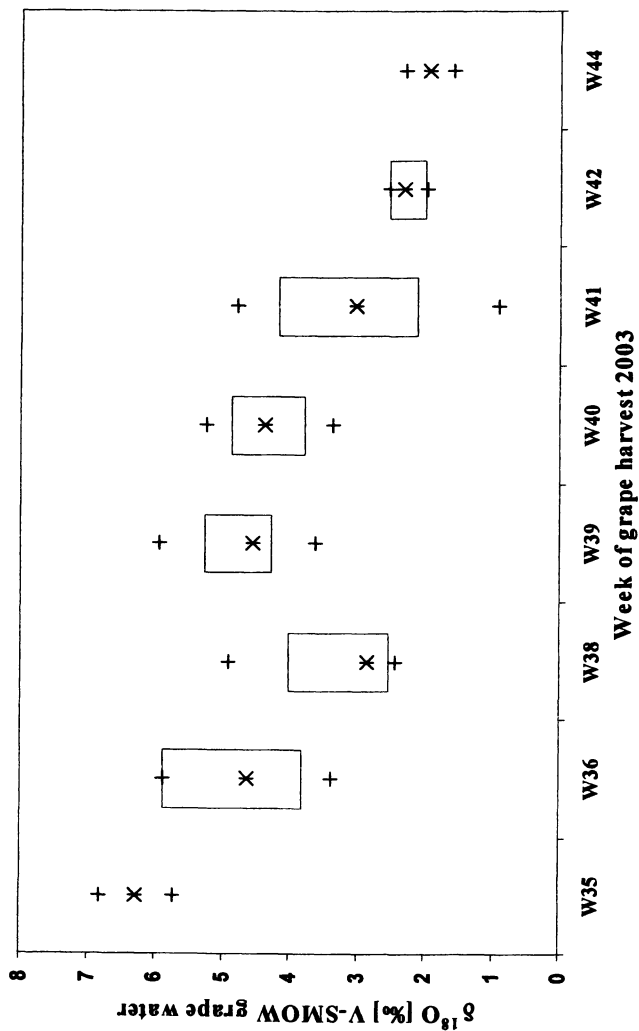


Figure 2. Influence of the date of vintage on $\delta^{18}\text{O}$ -values of water by example of authentic wines, Franconia 2003

regions due to higher precipitation and lower temperatures. Discussing authentic wines from Croatia, it was shown that this country is a typical example of extreme climatic differences in the same country (21). The dry and hot climate in Coastal Croatia caused extreme $\delta^{18}\text{O}$ -values up to +9 ‰ SMOW (mean +7 ‰) and (D/H)_{II}-values up to 134 ppm in 2000; also very high (D/H)_I-values (maximum 107 ppm) and $\delta^{13}\text{C}$ -values (maximum -23.1 ‰ V-PDB) were determined for these wines, which can only be measured for extremely dry and water-stressed sites. The isotope data of wines from Continental Croatia, a region with significantly lower temperatures and higher precipitation in 2000, are in the same range of isotope values of commercial wines from Macedonia, Yugoslavia, Romania, and Bulgaria. To summarize, Figure 3 confirms that basically $\delta^{18}\text{O}$ - and (D/H)_{II}-correlation can be used for authentication of geographic origin and year of vintage. In this context it is important to refer to corresponding data of Croatian wines of 2001 (21) which confirm that for interpretation of isotope data of wines from southern European regions also micro-climatic parameters and special meteorological influences have to be taken into consideration; for example untypical low (D/H)_{II}-values of ethanol and $\delta^{18}\text{O}$ -values of water were found in single authentic wines from one specific region in Coastal Croatia, which were caused by high precipitation (up to 190 l/m²) within four weeks before grape harvest.

Table IV summarizes stable isotope data of some selected wines originating from USA (California), Australia, South Africa, New Zealand, Chile, and Argentina; the wines were taken by official controllers in retail shops in 2003 and 2004. Most significant differences can be observed for the (D/H)_I-values of ethanol and $\delta^{18}\text{O}$ -values of water, whereas the $\delta^{13}\text{C}$ -values vary only between -28 to -26 ‰ V-PDB. Wines of Argentina are characterized by the lowest stable isotope ratios; their depletion can be explained by temperate continental climate, a relative high altitude of the vineyards (600-1300 m), and irrigation with extremely depleted water originating from the Andes. However wines from wine-growing regions of Chile which mostly are located in the same latitude and altitude and which are also irrigated with water from the Andes, have significantly higher (D/H)_I-, (D/H)_{II}-, and $\delta^{18}\text{O}$ -values. Since these differences between wines from Chile and Argentina cannot be explained by climatic effects or irrigation, further research and traceability of viticultural and oenological practices in both countries would be necessary. The highest isotope ratios were determined in wines from South Africa and Australia due to the rather hot and dry climate. The isotope pattern of Californian wines is almost similar to wines from South Italy, whereas the pattern of New Zealand wine is typical for a climate similar to Continental Europe. Since all data in table IV were within the statistical variation of corresponding data of the 'Third Countries Databank' and data published in literature (23), the wines were assessed to be authentic.

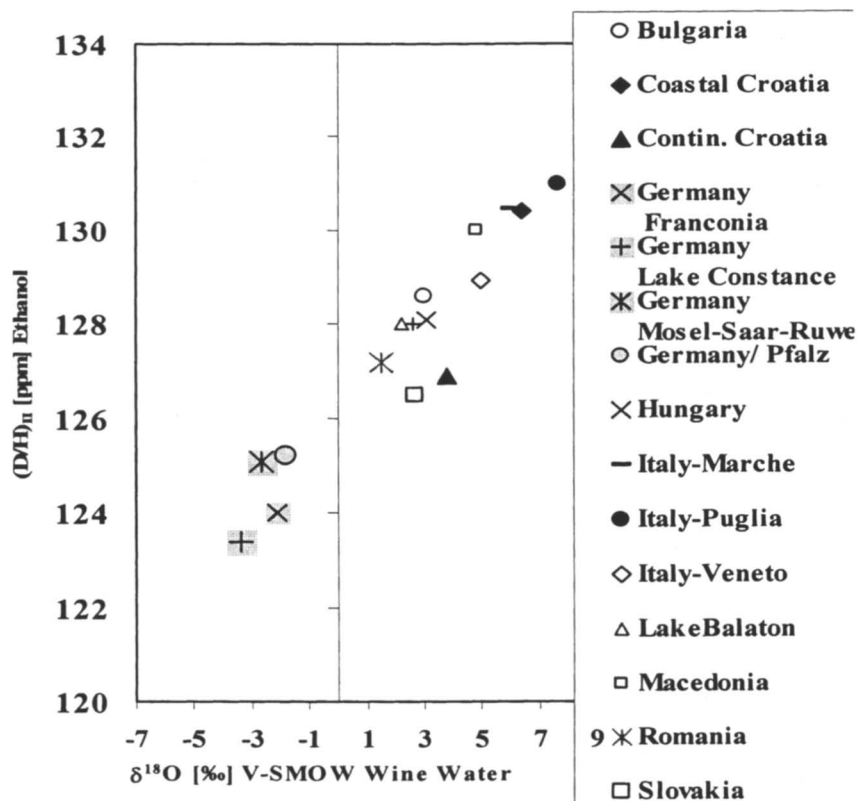


Figure 3. Mean $\delta^{18}\text{O}$ -values of water and $(D/H)_{II}$ -values of ethanol of authentic wines and commercial samples (Macedonia, Romania, Slovakia, Bulgaria) of different geographic origin, vintage 2000

Table IV: Stable isotope data of some selected wines originating from different continents, taken from German market in 2003/2004

Country	<i>N</i>	$\delta^{18}\text{O}$ ‰ SMOW wine water	$(D/H)_I$ ppm ethanol	$(D/H)_{II}$ ppm ethanol	$\delta^{13}\text{C}$ ‰ PDB wine ethanol
California	3	4.9	102	129	-27.5
South Africa	3	7.5	106	131	-26.5
Australia	2	7	105	132	-27
New Zealand	2	1.5	102	130	-28
Chile	5	4.5	102	130	-27
Argentina	3	0.5	98	125	-27

$^{13}\text{C}/^{12}\text{C}$ isotope ratio of sugar and ethanol – an indicator of soil water status

The $\delta^{13}\text{C}$ -values of ethanol of wines from southern European regions are normally in a range between -24 to -26 ‰ V-PDB, whereas wines from northern wine-growing areas of Europe, like Germany or Northern France have $\delta^{13}\text{C}$ -values between -27 and -29 ‰; the reason for the differences between southern and northern wine-growing regions in Europe can be put down to the fact that the $^{13}\text{C}/^{12}\text{C}$ -isotope ratio of sugar and ethanol is influenced by the vine water status; the variation of $\delta^{13}\text{C}$ -values of wines from Franconia and Lake Constance (Table II and III) prove this influence by a significant correlation of the $\delta^{13}\text{C}$ -value with the mean precipitation during period of grape ripening.

The significant effect of a decrease of $\delta^{13}\text{C}$ -value of ethanol in wines originating from regions or years with higher precipitation are also confirmed by the investigations of Gaudillère et. al (22) who found that the $\delta^{13}\text{C}$ -value of must sugar can be used to characterize vineyards for their soil structural capacity to provide water to grapevines and to detect water stress which is a result of climatic conditions. Gaudillère also found that the $\delta^{13}\text{C}$ -value allows a comparison of the effect of water stress response on grapevines, since water stress changes water use efficiency and carbon isotopic composition. In order to examine these effects on isotope fractionation of other isotope ratios, selected wines which were produced from a series of experiments on the influence of plant water status on grape quality by the 'Geisenheim Research Institute' (24) were analyzed by SIRA. Figure 4 shows by example of Riesling wines from the vineyard site 'Rüdesheimer Schlossberg' not only the significant influence of the year but also the effect of water stress and irrigation on carbon and deuterium stable isotope composition of ethanol. With the exception of a small area, this vineyard is characterized by a high risk of water stress. The $\delta^{13}\text{C}$ -values of ethanol of the wines produced from grapes of the water-stressed areas of the vineyard are in both years significantly more positive compared to those of the irrigated parts of the vineyard and the area with lower water stress. The (D/H)_I-ratio of ethanol shows in 2002 a different behaviour than in 2003; in 2002 the (D/H)_I-value of wine from grapes with lower water stress is higher than the those of the irrigated and non irrigated grapes whereas in 2003 the (D/H)_I-value of the wine from the non-irrigated area is significantly higher than the other variants.

Evaluating the mean values of $\delta^{18}\text{O}$ -values, all the samples did not show a significant variation in 2002 (1.1 ‰ V-SMOW +/-0.2 ‰) or in 2003 (3.4 ‰ +/- 0.4 ‰); it was also surprising that for (D/H)_{II}-values no significant variation could be observed in 2002 (122 - 123 ppm) and 2003 (124.9 ppm +/- 0.2). These results may be explained by the fact that vines were only irrigated with 71.5 L/vine (26 L/m²) in 2002 und 104 L/vine (37.8 L/m²) in 2003, and a temporal distance of 8 weeks between grape picking and the last irrigation. Further studies and more data are necessary to verify and quantify known effects

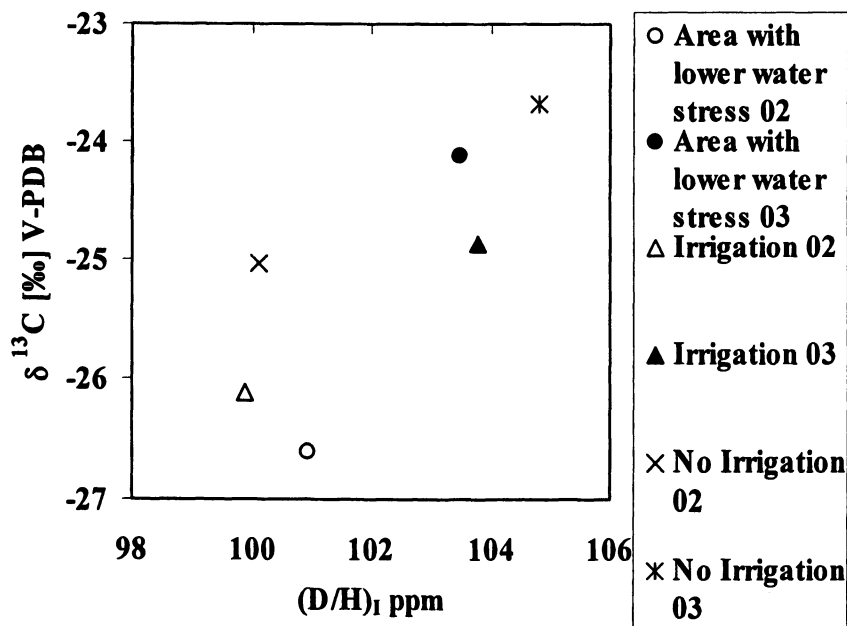


Figure 4. Examples for the influence of irrigation and water deficit on carbon and deuterium stable isotope composition of ethanol of Riesling wines from two different vintages (2002/2003) Vineyard site: 'Rüdesheimer Schlossberg'

of soil water status, irrigation, etc. on isotope fractionation (18,19,22) of different elements and components.

Summary

Multi-element and multi-component stable isotope ratio analysis can be used for authentication and traceability from vineyard to retailer and consumer, since stable isotope fractionation of carbon, hydrogen and oxygen integrate both biotic and abiotic factors with the result of an isotope pattern which is typical for geographic origin, year of harvest, climate, and raw material. Further research on special effects which may influence isotope fractionation are necessary especially the integration of other isotope ratios (⁸⁷Sr/⁸⁶Sr, ¹⁵N/¹⁴N, ³⁴S/³²S) which are already used for authentication of other food.

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Chapter 12

A Simplified Approach to Wine Varietal Authentication Using Complementary Methods: Headspace Mass Spectrometry and FTIR Spectroscopy

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Two methods for wine varietal authentication are evaluated using two complementary analytical approaches. A mass spectrometer is used to detect the headspace volatiles and fourier transform infrared (FTIR) spectroscopy is used to measure the phenolic compounds of four red and four white wine varietals from 18 different wineries across the United States. The spectra generated by each technique is analyzed by chemometric techniques to differentiate between the varietal wines. K-Nearest Neighbor (KNN) and Soft Independent Modeling of Class Analogy (SIMCA) models are developed for varietal authentication. Partial Least Squares (PLS) models are developed to model the binary mixtures between varietal wines and to determine the contributions of the varietals in wine mixtures. Chemometric models based on the MS volatiles spectra and the FTIR phenolics spectra offer a simple and fast approach to analyze and classify the varietal nature of wines.

Introduction

The character and quality of a wine is dependant on the grape varietal used in the manufacturing procedure. Understanding the differences between varietals is becoming extremely important in wine consuming countries where there is an opportunity for fraud and premium prices are paid for excellent quality products. Consumers place a great deal of trust on labels as to the composition and quality of the product. A fraudulent producer could gain advantage in a market place by providing incomplete or misleading information about their product.

Grape varietal adulteration is defined as adding must made from grapes originating from varieties other than the labeled varietal in quantities exceeding the limits specified by law. In the United States of America, at least 75% of the wine must be derived from grapes of the variety specified on the label (1). Currently there are no methods available to effectively distinguish between different varietal wines.

To differentiate between wine varietals, various approaches have been studied: analysis of the volatile content (2,3), amino acid profiles (4,5), protein fractions (6,7), physical parameters (8), and phenolic compounds (9-16). Although these methods provide valuable information, most of them are time consuming and do not provide conclusive information for varietal authentication. Recently, efforts have been made in our laboratory using a different approach to explore wine varietal authentication. As an alternative to measuring for specific components, a set of spectra measuring multiple components, is generated and analyzed by chemometric algorithms to differentiate between varietals. All compounds that contribute to the varietal uniqueness of the wine within the spectra are included in the analysis of the wine varietals.

Currently, two techniques are being used in our laboratory to measure complementary aspects of wine varietals. The first method uses headspace mass spectrometry and chemometrics to profile the volatile components of wine varietals. It is well known that different varietal wines contain different composition of aroma or volatile components. The second method uses mid-range FTIR and chemometrics to profile the phenolic compounds of varietal wines. The type and amount of the phenolic compounds are very important to the overall chemical composition of a wine. They are the major contributor to the taste and color of wines and are responsible for the stability of wines as they age. Detailed descriptions of the phenolic compounds are discussed in a previous review (10). The techniques presented do not require extensive sample preparation procedures to measure the spectra. We report here the findings of the two approaches for wine varietal authenticity.

Materials and Methods

Wine Samples.

Wines of eight pure varieties were collected from various wineries across the USA from the 1998, 1999 and 2000 harvest year. Each wine was certified at the winery by TTB investigators as being 100% pure varietal. For each grape varietal, the wines were collected from different wineries (Table 1).

Table 1: Description of the 100% Varietal Wine Samples

Four Red Wines	
Merlot (ME)	4 different wineries
Pinot Noir (PN)	5 different wineries
Cabernet Sauvignon (CS)	4 different wineries
Zinfandel (ZN)	4 different wineries
Four White Wines	
Sauvignon Blanc (SB)	4 different wineries
Chardonnay (CH)	4 different wineries
Riesling (RE)	5 different wineries
Pinot Gris (PG)	4 different wineries

Measurement of the Volatile Content Using Headspace Mass Spectrometry.

Sample Preparation:

For each wine sample, the following sample preparation steps were followed:

1. The wine bottles were soaked in ice water for at least 10 minutes before opening the bottle to minimize the volatile content escaping from the wine. Then 5 mL of wine sample was transferred into a 10 mL vial, and the vial was immediately sealed by crimping the vial.
2. Ten (10) replicate samples were prepared for each bottle of wine.
3. For blending experiments, mixed samples from two different bottles of wine were prepared at varied mixing ratio.
4. Test samples were prepared by another chemist from the original set of wines. They included pure varietal wines and binary mixtures of the varietal wines.

5. The samples were analyzed on the same day by a Gerstel headspace mass spectrometer ChemSensor (Gerstel Inc, Baltimore, MD).

Headspace Analysis:

Measurement of each sample was performed under the following conditions: incubation temperature: 85°C, sample incubation time: 20 minutes, syringe temperature: 90°C, injection port temperature: 180°C and MSD interface temperature: 200°C, injection volume: 1 mL, split mode: splitless for 0.5 minutes. The temperature was increased steadily from the incubation chamber to the MSD interface to prevent the sample volatile constituents from being condensed at each step.

The sample was introduced into the ionization source without prior separation with a scan range of 50-150 amu. All the mass spectra obtained for a wine sample were accumulated to generate a composite mass spectrum. This composite mass spectrum represents a fingerprint of the wine. Figure 1 shows a typical composite mass spectrum of a wine sample. To avoid the interference from ethanol, which generates ions at 45 and 46 amu, the scan range was started at 50 amu. Since there were no major ions produced from wine samples beyond 150 amu, the scan range was set up from 50 to 150 amu.

Pirouette (version 3.11, infometrix, Bothell, Washington) was used to process the mass spectrum data. The data was preprocessed by vector normalization. Two classification models, K-Nearest Neighbor (KNN) and Soft Independent Modeling of Class Analogy (SIMCA) were developed using the composite mass spectra of the wines.

Measurement of the Wine Phenolics Using FTIR.

Phenolic Extract Preparation:

To measure the phenolic profiles of the wines it was important to pre-treat the samples. The absorptions from ethanol, carbohydrates, water, and organic acids will mask the absorptions from phenolic compounds if left untreated (Figure 2). Solid phase extraction (SPE) using C-18 sorbent has been shown to be an effective phase to separate the phenolic components from other interferences in wine (17).

All wine samples were filtered before SPE with a disposable syringe filter (0.45 μ m pore size). Bond Elute solid phase cartridges (Varian, Walnut Creek, California), containing 1000 mg of C-18 sorbent in a 3 mL cartridge, were

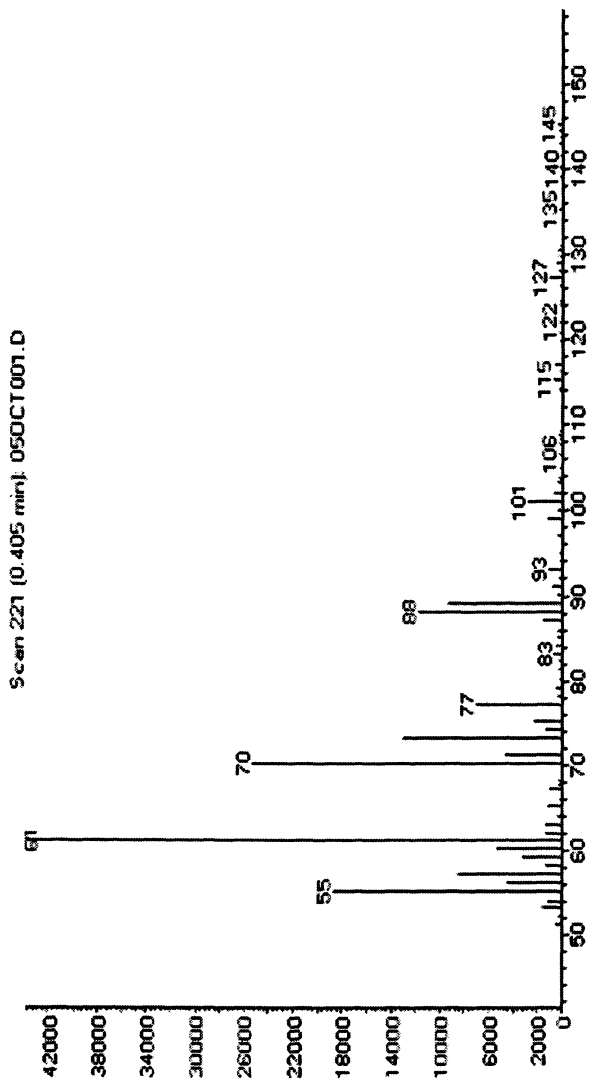


Figure 1: Typical Composite Mass Spectrum

preconditioned with 10 mL of methanol and 15 mL of distilled water (14). Three mL of red wine or 6 mL of white wine were loaded onto the column. A higher volume of white wine was used to concentrate the phenolics. The cartridges were then washed with 20 mL of deionized water. The extracts were eluted with six portions of 0.5 mL of acidic methanol (0.01% HCL). The SPE procedure was automated with a Rapid Trace SPE Workstation (Caliper Life Sciences, Massachusetts, USA).

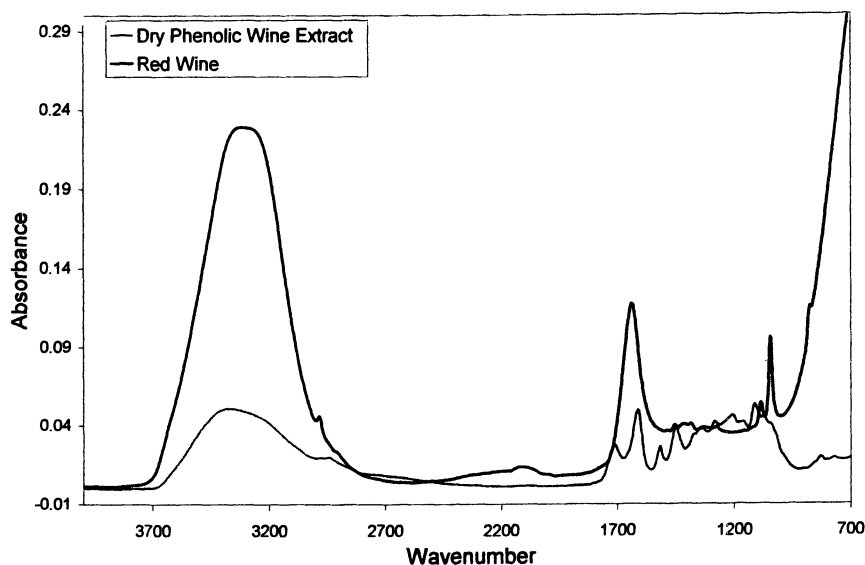


Figure 2. Spectrum of a red wine sample and phenolic extract

Measurement and Analysis of Phenolic Profiles:

Measurements were collected using a Bruker Tensor 37 FTIR (Billerica, Massachusetts) equipped with a single bounce attenuated total reflection (ATR) from SensIR (Danbury, Connecticut). Data was collected over the range from 4000 – 700 cm^{-1} , at a resolution of 4 cm^{-1} (300 scans, apodization function: Blackman-Harris-3-term). A Spectrum of the clean, dry ATR crystal against air was used for the background. One μL of red wine phenolic extract was transferred onto the ATR crystal and allowed to dry. For the white wines, a total of 2 μL was used, 1 μL was added and allowed to dry before an additional 1 μL was added. Spectra were recorded after complete drying of the samples. Four replicate spectra were recorded for each of the pure varietal wines from the same bottle, and two replicate spectra were recorded for the selected mixtures of the wines and test samples.

Analysis of the FTIR spectra was carried out using chemometric software Pirouette. Samples were preprocessed by taking the first derivative (Savitsky-Golay algorithm, 9 points) and vector normalization. Principal Component Analysis (PCA) scores plots were used to visualize the separation of the wine varieties. K Nearest Neighbors (KNN) and Soft Independent Modeling of Class Analogy (SIMCA) models were developed to model the differences between the grape varieties. Separate models were created for red and white wines. Because of the small sample size, leave-one-out validation procedures were used to validate the models. The models were used to classify test samples consisting of 100% pure varietal samples and binary mixtures of pure varieties. Partial Least Squares (PLS) models were created to demonstrate the regression between different concentrations of varieties within blended samples.

Results and Discussion

Modeling of Wine Varietals by the Volatile Content.

The mass spectrum obtained is a composite of all fragmented ions derived from various components in the wine vapor without prior separation. Two Chemometrics classification models, K-Nearest Neighbor (KNN) and Soft Independent Modeling of Class Analogy (SIMCA), are developed using the composite mass spectra of the wines. In Figure 3, a plot of three-dimensional SIMCA class distances for white wines is shown. Each wine sample is replicated 10 times. The four white varietal wines form separate clusters with a certain distance separate from each other. The interclass distances between individual clusters is greater than three, which indicate an adequate separation between the wines (18).

In Figure 4, the three-dimensional KNN class fit plot of four white wines is observed, which forms four separated clusters. But, some overlapping between Riesling and Sauvignon Blanc at the bottom region of the plot, and between Pinot Gris and Chardonnay at the top is observed.

Both KNN and SIMCA models are established as preliminary models. Test samples, prepared by a second chemist from the original set of wines, are classified by both models. The results are listed in the Table 2. Both models classify all 100% pure varietal test samples correctly, however, further testing of the model is necessary with wines from outside the validation set in order to fully test this method. Vintage and manufacturer are also considered but did not have a significant effect on the model.

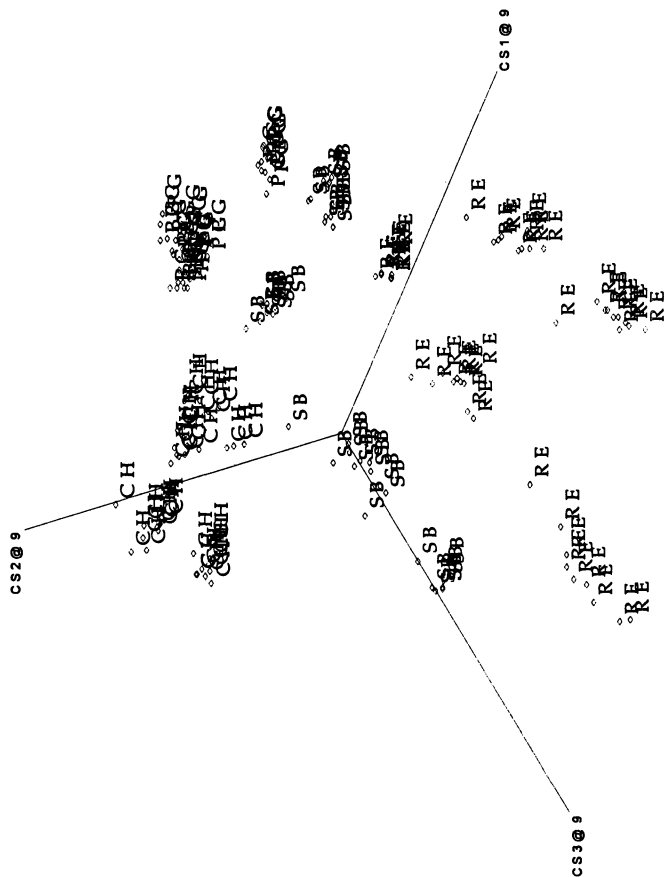


Figure 3. Three-dimensional SIMCA class distance for Riesling (RE), Pinot Gris (PG), Chardonnay (CH) and Sauvignon Blanc (SB) using volatiles data.

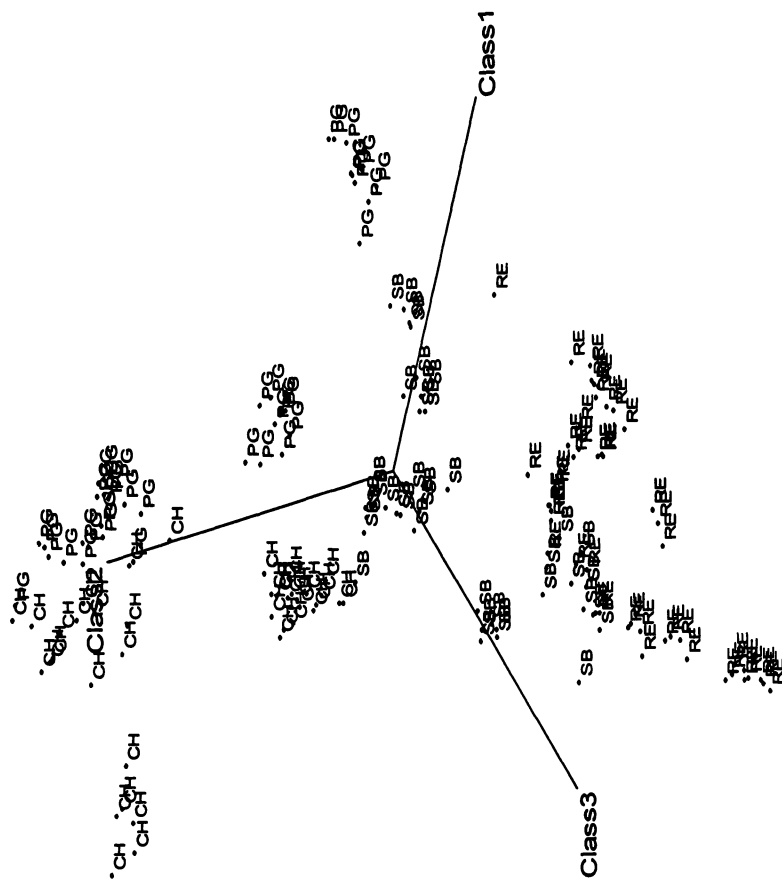


Figure 4. Three-dimensional KNN class fit for Riesling (RE), Pinot Gris (PG), Chardonnay (CH) and Sauvignon Blanc (SB) using volatiles data.

Table 2: Classification of white wine test samples from volatiles analysis

<i>Test Sample</i>	<i>KNN Prediction</i>	<i>SIMCA Prediction</i>	<i>Actual</i>
1	PG	PG	100 PG
2	CH	CH	100 CH
3	CH	CH	100 CH
4	RE	RE	100 RE
5	CH	CH	100 CH

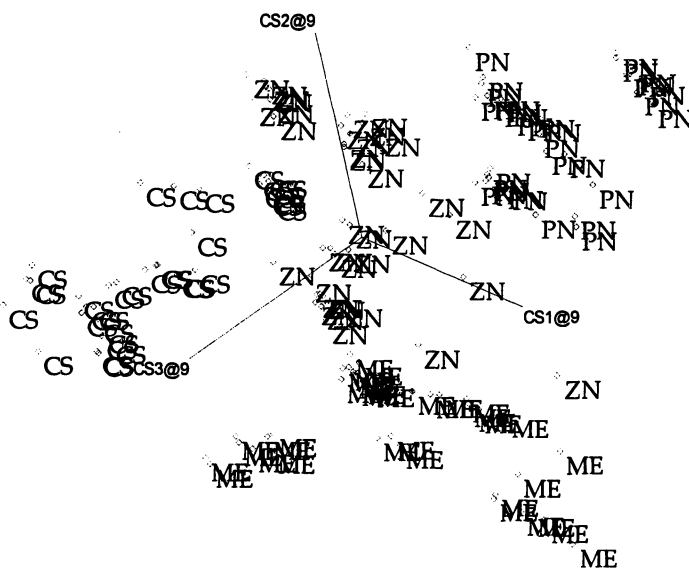


Figure 5. Three-dimensional SIMCA Class Distance for Cabernet Sauvignon (CS), Zinfandel (ZN), Merlot (ME) and Pinot Noir (PN) from volatiles analysis.

In Figure 5, a plot of the three-dimensional SIMCA class distances for red wines is shown. Four red varietal wines form separate clusters with certain distance from each other. The interclass distances between individual clusters is greater than three, which indicate a good separation between the wines.

In Figure 6 is the three-dimensional KNN class fit plot of four red wines. Zinfandel slightly overlaps with other three wine varieties. Merlot, Pinot Noir, and Cabernet Sauvignon form separate clusters.

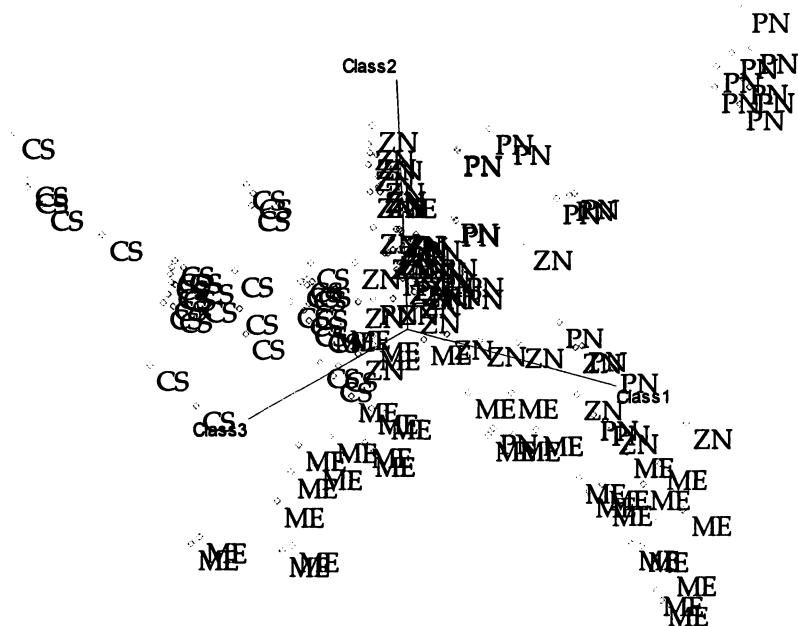


Figure 6. Three dimensional KNN class fit for red wine samples using data from volatiles analysis. Cabernet Sauvignon (CS), Zinfandel (ZN), Merlot (ME), Pinot Noir (PN).

The KNN and SIMCA models of the red wines are used as preliminary models, their suitability is tested to classify some red wine samples without knowing the identities of the samples at the time of testing. The classification results are listed in Table 3. The classification rate for the KNN and SIMCA models is 92% and 83% respectively.

Modeling of Wine Varietals by the Phenolic Components.

In Figure 2, the absorptions of interest in the mid-infrared region are detected from 1800 cm^{-1} to 800 cm^{-1} . The phenolic profile in this region of the

Table 3: Classification of red wine test samples from volatiles analysis

<i>Test Sample</i>	<i>KNN Prediction</i>	<i>SIMCA Prediction</i>	<i>Actual</i>
1	ZN	ZN	100 ZN
2	PN	PN	100 PN
3	CS	CS	100 CS
4	CS	CS	100 CS
5	ME	ME	100 ME
6	ZN	ZN	100 ZN
7	CS	CS	100 CS
8	ZN	ZN	100 ME
9	PN	PN	100 PN
10	CS	CS	100 CS
11	ME	ME	100 ME
12	PN	ME	100 PN

NOTE: Samples that were incorrectly predicted are in bold.

spectrum is heavily influenced by the aromatic nature of phenolic compounds. It is impossible to label specific absorptions within the spectrum because of the complex and diverse nature of the phenolic compounds. However, between 1800-1500 cm^{-1} there are strong absorptions from mono and di-substituted carbon compounds. In the middle region of the spectrum, from 1500-1250 cm^{-1} , are hydrocarbon bending vibrations that influence the spectra. Other important vibrations include O-H phenol vibrations from 1200-1000 cm^{-1} . The composition of phenolic compounds differs among each varietal leading to a different absorbance pattern for each varietal.

Discrimination of the red wine varietals is achieved by analysis of the phenolic profiles for each wine. Principal component analysis (PCA) scores plots are analyzed to visualize separation between the varietals. Factors 1 and 2 describe the difference between Pinot Noir and the other three varietals. Discrimination of all four varietals is illustrated in factors 3, 4, and 5 (Figure 7). In this plot, the wines cluster in regions according to the wine varietal. The separation is optimized by using the FTIR spectrum range 1800-1000 cm^{-1} . KNN and SIMCA models are developed to model the separation of the varietals. The KNN model is developed using the three nearest neighbors.

The models are validated according to the leave-one-out approach. In this approach a model is created excluding one of the samples. The model created is used to predict the sample left out. This procedure is repeated for each sample in the data set. The results are shown in Table 4. Merlot, Pinot Noir, and Zinfandel are well separated according to the results. Two of the Cabernet Sauvignon

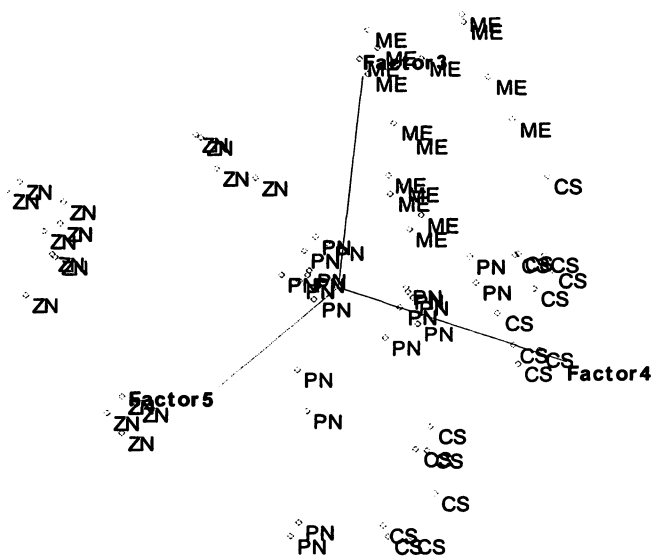


Figure 7. Three dimensional PCA scores plot for Cabernet Sauvignon (CS), Zinfandel (ZN), Merlot (ME), and Pinot Noir (PN) using phenolics data. Variance are 8%, 6%, and 3% for factors 3, 4, and 5 respectively.

Table 4. Leave one out validation results for the red wine KNN and SIMCA models using phenolics data

<i>Varietal</i>	<i># of samples</i>	<i>KNN (3)</i>	<i>SIMCA</i>
Cabernet Sauvignon	4	2	2
Merlot	4	4	4
Pinot Noir	5	5	5
Zinfandel	4	3	4

NOTE: Number of samples predicted correctly.

samples are not classified correctly according to the validation method. The effects of the vintage year and the manufacturer are investigated as reasons for the misclassifications, but no conclusions could be drawn. More samples are needed to better model the variations within Cabernet Sauvignon.

To assess the models, test samples are classified with the red wine models. The test samples, are prepared by a second chemist from the original wines, and include pure varietal wines and selected mixtures of the pure varietals. The results from the test samples are shown in Table 5. All of the pure varietal samples are correctly classified, as well as the majority of the mixed samples. These results provide preliminary evidence that the models will be able to predict the major grape varietal in wines that are not 100% pure varietal, however more work with a larger number of samples is needed.

Table 5. Classification of red wine test samples using phenolics data.

<i>Test Sample</i>	<i>KNN (3) Prediction</i>	<i>SIMCA Prediction</i>	<i>Actual</i>
1	ZN	ZN	100 ZN
2	ZN	ZN	100 ZN
3	ME	ME	100 ME
4	ME	ME	100 ME
5	ME	ME	100 ME
6	CS	CS	100 CS
7	PN	PN	100 PN
8	ZN	ZN	100 ZN
9	ZN	ZN	80 ZN / 20 CS
10	PN	PN	80 PN / 20 CS
11	ME	ME	75 ME / 25 ZN
12	CS	CS	75 CS / 25 ZN
13	PN	PN	75 PN / 25 ZN
14	ME	ME	75 ME / 25 ZN
15	ZN	ZN	75 ZN / 25 PN
16	ME	ME	75 ME / 25 PN
17	CS	CS	75 CS / 25 ME
18	PN	PN	75 PN / 25 ME
19	PN	PN	75 PN / 25 CS
20	ZN	ZN	75 ZN / 25 PN
21	ZN	ZN	75 ZN / 25 CS
22	CS	ZN	75 CS / 25 ZN

NOTE: Samples that were incorrectly predicted are in bold.

Discrimination of the white wine varieties is achieved through analysis of the phenolic spectra. As a comparison to red wines, white wines have a lower amount of total phenolics because the fermentation of the wines does not involve the seeds and skins. A lower phenolic content decreases the absorbance in the mid-infrared region making it more difficult to record the specific profile of each variety. To increase the sensitivity, 2 μL of extracted phenolic is used for white wines as compared to 1 μL for red wines. KNN and SIMCA models are prepared in accordance with the red wine models and are validated with the leave-one-out approach (Table 6). The Riesling and Sauvignon Blanc varieties are well separated according to the validation results. Analysis of the validation of Chardonnay and Pinot Grigio shows a small separation, which results in several misclassifications for the two varieties.

Table 6. Leave one out validation results for the white wine KNN and SIMCA models using phenolics data.

<i>Varietal</i>	<i># of samples</i>	<i>KNN (3)</i>	<i>SIMCA</i>
Chardonnay	4	2	3
Pinot Grigio	4	2	2
Riesling	5	4	4
Sauvignon Blanc	4	4	3

NOTE: Number of samples predicted correctly.

Test samples are classified with the white wine models. The test samples include pure varietal samples and selected mixtures of the pure varieties. The test mixtures consist of 75% of one variety and 25% of a second variety. The results are shown in Table 7. Only two of the test samples are 100% varieties and both of the samples are predicted correctly. The mixed samples had different results for the two models. The SIMCA model classified more samples correctly than the KNN model. One possible reason for some of the misclassifications is due to an increase in sample variation within the white samples. The variation is a result of the low phenolic content and the extra steps that are needed to record a spectrum. However, the majority of the blind samples are classified correctly providing support that the model will have the ability to predict white wine varieties.

Prediction of Varietal Contributions in Blended Wines.

Estimating the percentage of a variety in a mixed wine sample is possible by measuring either the headspace or phenolic profile and using multivariate calculations. To demonstrate this, the phenolic profile of a set of three wines (Zinfandel, Pinot Noir, and Merlot) is measured and processed by partial least squares (PLS) algorithms. The projections of these pure wines, along with their binary mixtures (75/25, 50/50, and 25/75 for each pair), into the 2 factor space is shown in Figure 8.

Table 7. Classification of white wine test samples using phenolics data.

<i>Test Sample</i>	<i>KNN (3) Prediction</i>	<i>SIMCA Prediction</i>	<i>Actual</i>
1	PG	PG	100 PG
2	SB	SB	100 SB
3	CH	PG	75 PG / 25 RE
4	CH	CH	75 CH / 25 RE
5	PG	PG	75 PG / 25 CH
6	CH	CH	75 CH / 25 PG
7	RE	RE	75 RE / 25 PG
8	RE	RE	75 RE / 25 CH
9	CH	RE	75 RE / 25 SB
10	PG	PG	75 PG / 25 RE
11	PG	SB	75 SB / 25 RE
12	SB	SB	75 SB / 25 PG
13	SB	SB	75 SB / 25 PG
14	CH	CH	75 CH / 25 PG
15	SB	PG	75 SB / 25 CH
16	RE	RE	75 SB / 25 RE
17	CH	CH	75 CH / 25 RE
18	CH	CH	75 CH / 25 RE
19	RE	RE	75 RE / 25 CH
20	RE	RE	75 RE / 25 SB
21	SB	SB	75 SB / 25 CH
22	CH	SB	75 SB / 25 RE
23	CH	CH	75 CH / 25 SB
24	CH	CH	75 CH / 25 PG
25	PG	CH	75 CH / 25 PG

NOTE: Samples that were incorrectly classified are in bold.

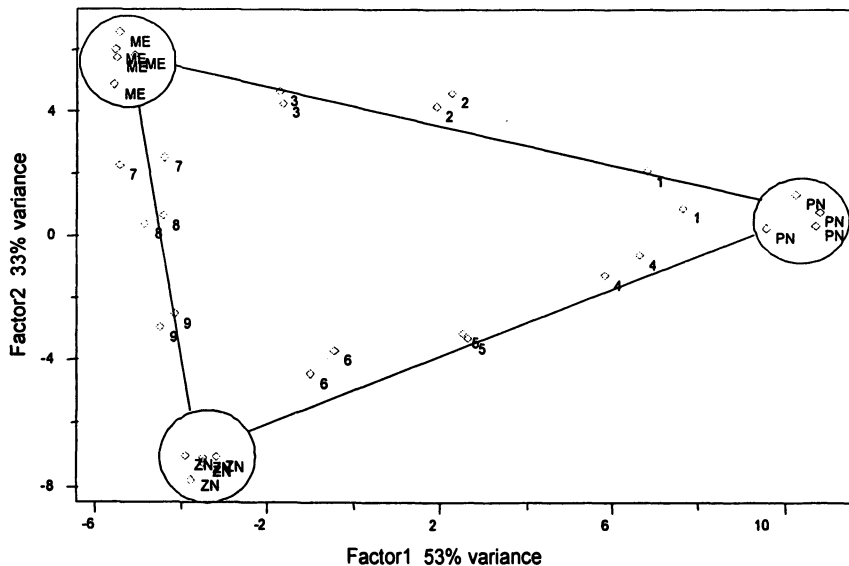


Figure 8. PCA scores plot of three wines of different varietals and binary mixtures of the varietal wines using phenolics data . Merlot (ME), Zinfandel (ZN), and Pinot Noir (PN).

Inspection of PCA scores plot (Figure 8) reveals a correlation between the pure varietal samples and the mixed samples. The three 100% varietals create a triangle, and the resulting binary mixtures form the sides of the triangle. Factor 1 is interpreted as the difference between Pinot Noir and the other two pure wines. Factor 2 is interpreted as the difference between Zinfandel and Merlot. The closer a mixed sample is to a pure varietal in factor space, the higher the concentration of the varietal in the mixed sample. For example, there are three ratios of Merlot and Zinfandel. The primary component in mixture 9 is Zinfandel, whereas the primary component in mixture 7 is Merlot. Mixture 8 is a 50/50 mixture of the two varietals. The same relationship holds for the other two sets of pure varietals.

The relationship between the varietal wines is modeled by PLS algorithms. Within the PLS model, a 5-point percentage plot, from 0 to 100 percent, is created for each varietal (Figure 9). The models are used to predict the varietal composition of unknown samples. Six samples, of unknown varietal

concentration, are predicted using the PLS models (Table 8). On average, the models from the volatile components and the phenolics are able to predict the major component within 5% of the actual content.

Conclusion

Discrimination of pure varietal wines is demonstrated with two different complementary analytical methods. Headspace mass-spectrometry profiled the volatile components and fourier transform infrared spectroscopy measured the phenolic compounds. Classification models (KNN and SIMCA) are created from the headspace volatiles that fully discriminate between the pure varietal wines. The models are then used to classify pure varietals of red and white wine. All the white wines are classified correctly and the majority of the red wines are classified correctly. The classification models for the phenolic spectra completely discriminate between the pure varietal wines. A higher degree of separation is observed in the red wine models which is attributed to the fact that red wines have more phenolics than white wines. Successful classification of the mixed samples provides confidence that the models will be able to identify wines that are not pure varietals. Future work is in progress to expand the number of pure varietal wines in the modeling set to account variations observed in the classification models.

PLS models are demonstrated as a powerful tool to classify the percentage of a varietal in a wine sample. Models are constructed from two pure varietal wines and binary mixtures of the two wines. The models correctly classify

Table 8. PLS classification of test samples using phenolics data

<i>Test Sample</i>	<i>Predicted Value</i>	<i>Actual Value</i>
1	101 ME	100 ME
2	75 PN / 25 ZN	75 PN / 25 ZN
3	40 PN / 60 ZN	50 PN / 50 ZN
4	40 ZN / 60 ME	50 ZN / 50 ME
5	80 ME / 25 ZN	75 ME / 25 ZN
6	71 ZN / 29 PN	75 ZN / 25 PN

NOTE: data listed is the varietal percentage

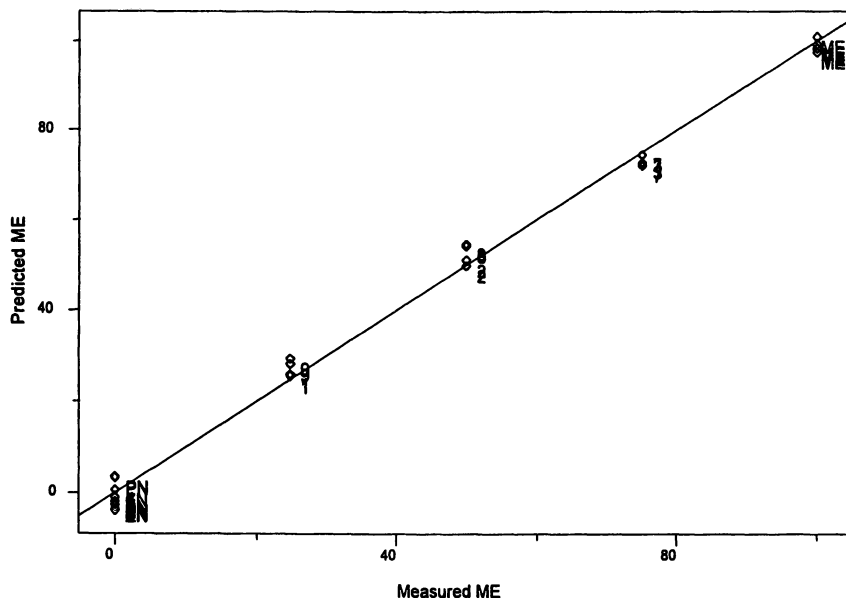


Figure 9. PLS model for the prediction of the % ME in an unknown sample using phenolics data.

samples composed of mixtures of the wines from the model. PLS models created from the spectrum of the phenolics compounds and volatile compounds identified the varietal concentration of blind samples with an average deviation of 5%

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Chapter 13

Geographic Origin of Wine via Trace and Ultra-Trace Elemental Analysis Using Inductively Coupled Plasma Mass Spectrometry and Chemometrics

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The use of trace and ultra-trace elemental mapping of wines along with chemometrics was successfully applied to authenticate geographical origins of wines. The elemental mapping of wines was carried out using microwave digestion of the solids in a known volume of a wine in concentrated nitric acid followed by ICP-MS analysis. The concentrations of ten trace and ultra-trace elements were determined in thirty four wines from four geographical locations within the USA. The wines were produced from grapes grown in the known geographical areas. The samples comprised of white and red wines of vintages not differing by more than three years. Multivariate analyses of the elemental profiles were conducted to develop models such as principal component analysis and soft independent modeling of class analogy (SIMCA) to classify the wines. The results showed that the combination of nine elements found in wines accurately classify the products according to their geographical origin. Work is in progress to collect more authentic samples from the locations tested so far into models to make them more rugged.

The wine industry shares many goals with other food industries: reducing the costs while maintaining or increasing the quality of their product and preventing counterfeit products from entering their markets. In recent years, the Alcohol and Tobacco Tax and Trade Bureau (TTB) of the Department of the Treasury has received several complaints of counterfeit products being sold in the market. Such counterfeit products are identified through chemical analyses in comparison with an authentic reference. The Alcohol and Tobacco Tax and Trade Bureau is interested in protecting consumers from deception as well as genuine producers from fraudulent competitors. In recent years, many research papers have appeared in journals showing the use of the concentrations of several organic and inorganic species and chemometric classification models in authenticating the geographical origins of wines (1).

The use of trace elements concentrations for determining geographic origins of wines has been successfully demonstrated by several scientists engaged in enological research. The concentrations of several elements, metallic and non-metallic, were monitored in wines in the Okanagan Valley, Canada and the data were used in chemometric models for identifying their geographical origins (2). This work resulted in the successful classification of wines according to their origin. Establishing a data bank for the trace metals profiles of wines of known geographic origin will be a good foundation on which authentication work could be built.

Data on organic compounds (polyphenols and anthocyanins) and trace metals have also been used to determine the origins of wines (3). A successful discriminant model was derived to differentiate Spanish rose wines. Characterization of the geographical origin of Italian red wines through elemental analyses and NMR data had been reported (4). Using chemometric methods such as principal component analysis (PCA), hierarchical clustering analysis (HCA), and discriminant analysis (DA), this research was able to differentiate wines from the North, South and Central regions of Apulia, Italy (4). Stable isotope ratios of hydrogen ($^2\text{H}/^1\text{H}$), carbon ($^{13}\text{C}/^{12}\text{C}$), and oxygen ($^{18}\text{O}/^{16}\text{O}$), and meteorological data were used to authenticate wines from wine-growing regions of Germany (5). Classification of white wines from four German wine-growing regions using the concentrations of 13 elements and pattern recognition techniques was reported by Gomez et al. (6). The uptake of trace elements by plants is controlled by many factors such as the climate, rain, the age of the plant, the root depth, the irrigation water, and soil pH (7). However, the clarification methods used by wineries employ adsorbing materials such as silica gel, bentonite, and diatomaceous earth; these adsorbants could add trace elements to the wine. Trace elements could reflect soil compositions and would be of great help in fingerprinting wines (8).

In this paper we report the mapping of red and white wines from four geographical locations (New York, Pennsylvania, Oregon, California) using 10 elements including three ultra-trace elements followed by chemometric analysis to develop classification models. These 10 elements are Ti, V, Cr, Mn, Rb, Sr, Ba, La, Ce, and U. The main goal of this study was to check the possibility of classifying wines on the basis of their trace elemental concentrations. This is a preliminary work whose viability will form the basis of a broader data bank for the authentication of geographical origins of wines.

Materials and Methods

Wine Samples

Wine samples of vintages ranging from 1999 to 2000 were collected from four geographical locations including New York, Pennsylvania, Oregon, and California. All wines were produced from grapes grown in their respective regions. Although the TTB regulations allow for the inclusion of fruit grown from outside of the stated region (upto 25%), without identifying the region, efforts were made to collect only those wines which were produced from 100% locally grown fruit. A total of 34 wines (17 red and 17 white) were used for this study as shown in Tables 1 and 2.

Sample Preparation

Exactly 50 ml of a sample was placed in a Teflon tube of 'MARS 5 XP-1500 Plus' digestion vessel (CEM Corporation, Matthews, NC) and evaporated in an oven at 100°C until the volatiles were removed. The residue was digested with

Table I. Description of the Wine Samples

<i>Four Red Wines</i>	
Merlot	4 different wineries
Pinot Noir	5 different wineries
Cabernet Sauvignon	4 different wineries
Zinfandel	4 different wineries
<i>Four White Wines</i>	
Savignon Blanc	4 different wineries
Chardonnay	4 different wineries
Riesling	5 different wineries
Pinot Gris	4 different wineries

Table II. Geographical Locations of the Wine Samples

<i>Location</i>	<i>Red Wines</i>	<i>White Wines</i>
California	9	8
Oregon	4	4
New York	2	4
Pennsylvania	2	1

10 ml of 30% nitric acid in the MARS 5 XP-1500 Plus digestion vessel under high pressure (1500 psig) and temperature (200°C). After cooling, the solution was transferred to a 10 ml standard flask and brought up to volume with deionized water. The solution was filtered through a 0.45µm nylon membrane in a syringe filter and analyzed with Micromass ICP-MS. Six replicate samples were prepared for each bottle of wine.

ICP-MS Analysis

Inductively Coupled Plasma Mass Spectrometry (ICP-MS) provides greater sensitivity and lower instrumental detection limits than any other rapid multi-element technique (8). In this study, a Micromass Platform ICP-MS (Waters, Milford, MA) was used for determining the trace elements selected for authentication. Because of its high sensitivity, this instrument was capable of detecting these elements at low parts-per-trillion levels. The use of hexapole collision cell (He + H₂) in the instrument eliminates most of the molecular interferences and enhances the sensitivity. A standard solution containing multiple elements (10 mg/L each) was obtained from Solutions Plus, Inc. (Fenton, MO) and was used for preparing calibration standards. Typically a blank and three solutions of varying concentrations (5, 10, 20 µ/L) were used. Calibration curves were generated for all elements. The precision for all of the data collected was less than 5% RSD.

Multivariate Data Analysis

Multivariate analysis of the data on elemental concentrations was carried out on using a Pirouette, version 2.7 chemometrics software (Infometrix, Inc., Woodinville, WA). Applying the classification algorithm called 'Soft Independent Modeling of Class Analogy (SIMCA), a classification model was created for the samples analyzed.

Results and Discussion

Elements for authentication

The choice of elements plays an important role in the authentication study. The elemental composition of the wine is thought to be related to soil composition where the grapes were grown (10). Though most of the elements found in the wine reflect the elemental composition of the soil in which the grapes were grown and so this is how even regions located close to each other may be able to be distinguished (10). The trace and ultra-trace elements were selectively chosen because they can be readily determined with ICP-MS with little or no matrix interference (11). The elements chosen for this study include the trace metals Ti, V, Cr, Mn, Rb, Sr, Ba, and the ultra-trace metals, La, Ce, and U.

Multivariate Data Processing

The overall range of concentration of elements for all the wine samples was wide. For example, a typical wine from New York could contain up to 1130 ppb of Rb, 40 ppb of Cr and 0.50 ppb of La. In order to remove these scaling differences between variables, we decided to preprocess the data by using autoscaling. Autoscaling removes differences in units between variables by removing the mean and dividing each element by the standard deviation of that variable. Once autoscaling has been performed, each variable in the transform matrix will have a mean of zero and unit variance.

We processed the data by normalizing it to the highest element concentration which was zinc. The concentration of zinc ranged from 1800 ppb to 2900 ppb. Using this normalization future instrument fluctuations could be accounted for and have a lesser effect in the ability of the chemometric model to make accurate predictions.

The projections of the samples into the space of the first three principal components are shown in Figure 1. It can be seen in this figure that four distinct clusters representing samples from the four geographical regions are formed. Replicate samples cluster well together and it appears that even though the wines were from different wineries and of different vintages, they do cluster by geographical region. The total variance captured with three principal components accounts for 89.82% indicating that the systematic variance of the data set was captured with just three PCs. A SIMCA model was also created with this data set.

In this study, the number of samples representing each geographic region is limited. Further study will include more authentic wine samples in the database to make the models more rugged and the data will be corrected for any elemental contributions from clarifying materials such as bentonite and silica gel.

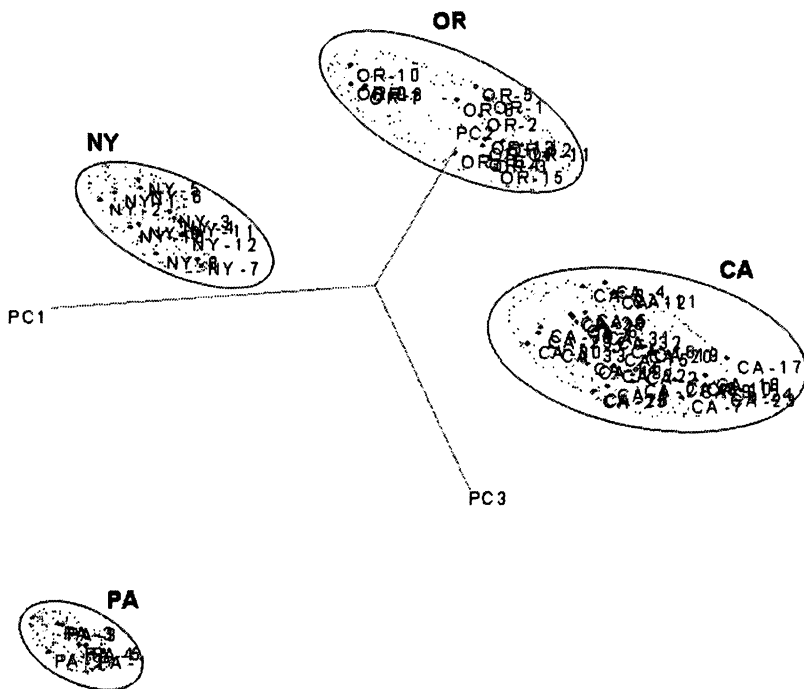


Figure 1. Projections of the elemental concentrations into the space of the first three principal components (89.82% of variance captured).

Conclusion

The concentrations of selected trace and ultra-trace elements in wines were used to develop classification models with multivariate analysis. Through preconcentration and acid digestion these elements can be readily determined at very low levels using an ICP-MS instrument. The study shows that the geographical origin of wines could be determined through their elemental compositions and chemometric classification models.

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Chapter 14

Grape and Wine Varietal Authentication by DNA Analysis

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The species *V. vinifera* L., is the main species used today for wine. It is a diverse species at the morphological level. In order to estimate the diversity of the nuclear and chloroplastic genomes, molecular markers have been developed. These markers can also be used to authenticate species and cultivars from a range of grape tissues. Today, the most popular markers in use by the grape community are the microsatellites. However, identification of clones from diverse cultivar backgrounds requires additional methodologies and remains difficult. Microsatellite markers have been used for the authentication of grape varieties from experimental wines but their application has not yet been possible for commercial wines. This chapter reviews the methodologies for analyzing DNA from grapes and wines and presents some of the limitations and challenges that remain in using this technology for authentication of grape species, cultivars, and clones from a range of grape tissues.

Introduction

World-wide grape production is extensive. According to the OIV 2005 report (<http://www.oiv.com>), the global production of grapes for 2004 was forecast to be 66 million tons (about 50 % from Europe, 20 % from Asia and 18.5 % from America). The main commercial products are table grapes, raisins and wine grapes (24%, 1.8 % and 74% of the world production, respectively). Rootstocks constitute another important product since most of the 8 million hectares of grape vineyards are grown as scions grafted on rootstocks.

Because many varieties exist for each of these four types of grape materials (see next section), each with very different characteristics, the identity of the material (with respect to genus, species, variety or cultivar, and clone) is crucial. Moreover, many regions of production, most noticeably the European Union, have very strict regulations concerning grape material identity. Therefore, the identification of plant material has become an important concern of the grape community and has received much attention from scientists.

The traditional method for identification, known as ampelography is based on visual comparisons of morphological characteristics (1), however ampelography is a difficult art and there are few experts and collections for comparison (2). Following forensic methods developed for humans, the possibility of analyzing DNA from grape material for identification purposes was proposed in the 1990s and lead to the development of active research work around the world. Several international initiatives (Genres081, IGGP) were proposed to improve the techniques and to set international standards for the identification of grape materials through the analysis of DNA.

In this chapter we present the different methods that have been developed, the possibilities offered in terms of identification, and a review of the most recent results on grape genetic diversity. In order to be complete and because 74% of the world grape production is marketed as wine, we will also review the present possibilities of identifying the grape cultivars within a wine for authentication purposes.

Grape Plant Material: A World of Diversity for a Meagre World Use

The commercial grape materials used today almost exclusively belong to the genus *Vitis* (with the exception of a few ornamental varieties). While *Vitis vinifera* L. is the most common species, about 13 American species have also extensively been used as pure species or for production of interspecific hybrids

because they are sources of resistances against foliar and root pests and diseases (3). Galet (4) compiled a list of hundreds of interspecific hybrids developed and used since the Phylloxera crisis. Because interspecific hybrids are presently forbidden in the EU, the production of hybrid grapes is limited to the Americas, Eastern Europe and the Far East.

The *Vitis* species are also extensively used for the development of rootstocks (3, 5). Since the introduction and worldwide spread of grape phylloxera, a root feeding aphid, the growth of *Vitis vinifera* cultivars on their own roots is indeed impossible in most parts of the world. The breeding of rootstocks in early 20th century, which are still used today, led to the development of hundreds of rootstock cultivars. However, only about 40 rootstock cultivars are used in today's vineyards.

Vitis vinifera L., the Eurasian grape, accounts for the vast majority of grape production. This species is extremely diverse, and about 5,000 to 6,000 cultivars are believed to exist (6). The actual number of cultivars is however only a small portion of this diversity, since only 300 to 400 cultivars are commonly used around the world (7). According to a 1998 estimation, only 44 cultivars represents 94% of the French vineyards. Most of the available genetic resources are therefore maintained in germplasm collections (OIV, 1987, <http://www.genres.de/vitis>), such as INRA Vassal domain (<http://www.montpellier.inra/Vassal/>), the most important germplasm collection for *Vitis vinifera* (about 5000 accessions).

These *V. vinifera* cultivars vary both in morphological (shoot tips, leaves, clusters, berries), and agronomical and technological characters (8, 9). Due to the long agricultural history of the species and the constant exchange of plant material between areas, a great number of synonyms among cultivars exist (6, 7) complicating identifications.

Although grapes are maintained clonally through vegetative multiplication, diversity also exists within the cultivars, termed clonal variability. Clonal variation occurs in many characters including yield and quality related traits (sugar content, berry size, perceived wine quality). This diversity is also more obvious in some cases, such as mutations that occur in berry color pathways. For example the "Pinot" family shows all possible berry color phenotypes: teinturier, black, red, grey, and white (7). Another important mutation is the presence of aromatic compounds such as in Gewürztraminer, an aromatic mutant of Traminer (also called Savagnin; 7).

In summary, the morphological diversity in *Vitis* is very important and it extends to the interspecific hybrids and rootstocks. Although the cultivars are propagated vegetatively, there is a small level of genetic diversity within the so-called clones, and these differences can occasionally be quite obvious among some important mutants. When the mutation concerns a trait of interest for the

final quality of the wine, these mutation-based clones are considered to be different cultivars (10).

Although the genetic diversity in grape is tremendous, only a small part of it has an economic relevance, which has restricted most analyses of genetic diversity to important cultivars. For analysis aimed at the confirmation of possible identities, (i.e., to differentiate unknown samples from a limited number of cultivars) this might be enough. However, for blind authentication of cultivars, the genetic diversity of a large number of cultivars must be analyzed before high confidence in the final results is possible.

Analyzing DNA from Grape: The Fingerprinting of the Vines

For humans, every individual has a unique DNA sequence and is therefore easily identifiable by the analysis of its DNA, which is then called DNA fingerprinting. DNA fingerprinting has been extensively used in forensic analysis (11). Similarly, genetic fingerprinting could be used for grape authentication, but its usefulness is determined both by the technique employed and the database of fingerprinted cultivars. After a brief review of the different fingerprinting methods developed for grape, we will analyze the level of diversity detected by different techniques and for different plant materials.

The Methods of the Past , Present and Future

Since the first analyses of DNA using the RFLP (Restriction Fragment Length Polymorphism) techniques (12-14), many additional techniques have been applied to the analysis of genetic diversity in grape. In this section, we will review some of these techniques and their advantages and limitations. All of them are based on the PCR (polymerase chain reaction) technique and they differ in the nature of the primers used for the amplification.

The first method that received much attention was the RAPD (Random Amplified Polymorphic DNA) method (Table I), based on the use of random 10mer-primers. This method has been applied to rootstocks (15) as well as scions (16-20), to the analysis of clonal variation (18, 21) and even the analysis of wild grapes (22) and species (23). Despite the limitations of this method (Table I), it is still used today (24). RAPD fragments can also be sequenced and transformed into SCAR (Sequenced Characterized Amplified Region) markers useful for the analysis of rootstocks (25) or scions (26).

Following the discovery of the value of SSRs (Simple Sequence Repeats) for grape analysis (27), this method became very popular in grape. The method uses short sequences (1 to 4 base pairs, Figure 1) repeated in tandem that can be

Table I. The Different Molecular Techniques Applied to the Analysis of Grape DNA

<i>Method</i>	<i>Acronym</i>	<i>Type of PCR</i>	<i>Need of development¹</i>	<i>Reproducibility between experiments</i>	<i>Transferability between lab</i>	<i>Diversity</i>	<i>Level of analysis proposed</i>	<i>Reference²</i>
Random amplified polymorphic DNA	RAPD	Random	Commercial kits	Limited	Limited	++	-	93, 94
Sequenced characterized amplified region	SCAR	Specific	Sequencing of RAPD	Good	Good	+	-	25*
Simple sequence repeats	SSR	Specific	Cloning and sequencing	Very good	Good	+++	Species, cultivars	27*
Inter SSR PCR	ISSR-PCR	Random	-	Fair	Limited	++	-	40*
Amplified fragment length polymorphism	AFLP	Random	Limited	Good	Limited	+++	Clonal	42

¹No need for any preliminary development, + to +++ indicates increased amount of development necessary

²Reference either of the initial paper or of the first use in grape (*)

amplified by PCR using specific oligonucleotides defined in adjacent sequences. Microsatellites are very abundant in the grape genome and many grape SSRs have been developed in non coding, as well as coding sequences (Table II). Because of the high transferability of the markers from one species to another, they have been developed either from *V. vinifera* or from other species, but can usually be easily used for the different *Vitis* species, and even for some *Vitaceae* species (28). The first international effort on grape genetics (*Vitis* microsatellite consortium coordinated by Agrogene), was launched by C. Meredith and led to the development of 150 SSR markers (Table II). Today more than 550 SSR markers are available, many of them being mapped on the grape genome (29-32). Amplified SSR fragments were first detected using slab polyacrylamide gels stained by silver-staining method (33; Figure 2A). This technique was replaced by more sensitive and convenient fluorescence detection coupled with capillary gel electrophoresis (Figure 2B).

Differences in SSR sizes are usually due to a variation in the number of repeats in the microsatellite, but they can also be the result of mutations in the flanking regions of the SSRs (34, 35). However, this type of variation is more common among species and cannot be detected by fragment sizing. Detection requires more sophisticated techniques such as sequencing and is therefore not compatible with high throughput analysis.

The use of SSR size variation has been a very useful technique for identification purposes, synonymy and homonymy description (Table III) as well as for parentage analysis (36-38). PCR amplifications of regions located between SSR sequences (Inter Simple Sequence Repeat PCR or ISSR-PCR) have also been applied to the analysis of grape DNA (39-41).

The most recent method is the AFLP (Amplified Fragment Length Polymorphism) method (42). This technique is based on the restriction of DNA using a frequent cutter restriction enzyme and the ligation of an adaptor corresponding to specific primers that will be used in the PCR amplification. Since many fragments result from the restriction, the amplified fragments are selected using a combination of additional bases on both primers. This technique has been used to characterize grape varieties and to distinguish clonal variation (43-46).

These methods have also been used in combination to increase the discrimination power. SSR and RAPD (18, 47, 48) as well as SSR and AFLP (49-55) have thus been combined. Although ampelography is traditional, it has been used in conjunction with molecular markers in grape diversity studies (46, 56).

Each of these techniques possess advantages and limitations (Table I) that reduce or enhance their use. In general, the PCR-based methods supplanted the RFLP method because they require limited amounts of DNA, which not only simplified the analysis but also enabled the analysis of single cell organs such as

Table II. List of the SSR Markers Developed for Grape

<i>Reference</i>	<i>Type of SSR</i>	<i># loci</i>	<i>Test of diversity</i>
95	Non coding	39	41 <i>V. vinifera</i>
96	Non coding	4	77 <i>V. vinifera</i>
97	Non coding	12	<i>V. vinifera</i>
98	Coding	8	<i>Vitaceae</i>
99	Non coding	118	<i>V. vinifera</i> and species
100	Non coding	7	<i>V. vinifera</i> and species
101	Non coding	169	<i>V. vinifera</i>
102	Non coding	7	<i>V. vinifera</i> and species
103	Coding	16	<i>V. vinifera</i> and species
104	Non coding	18	120 <i>V. vinifera</i> and species
27	Non coding	5	26 <i>V. vinifera</i>
SSR developed within VMC project	Non coding	150	<i>V. vinifera</i> , <i>V. riparia</i> , <i>Muscadinia</i>
Total		553	

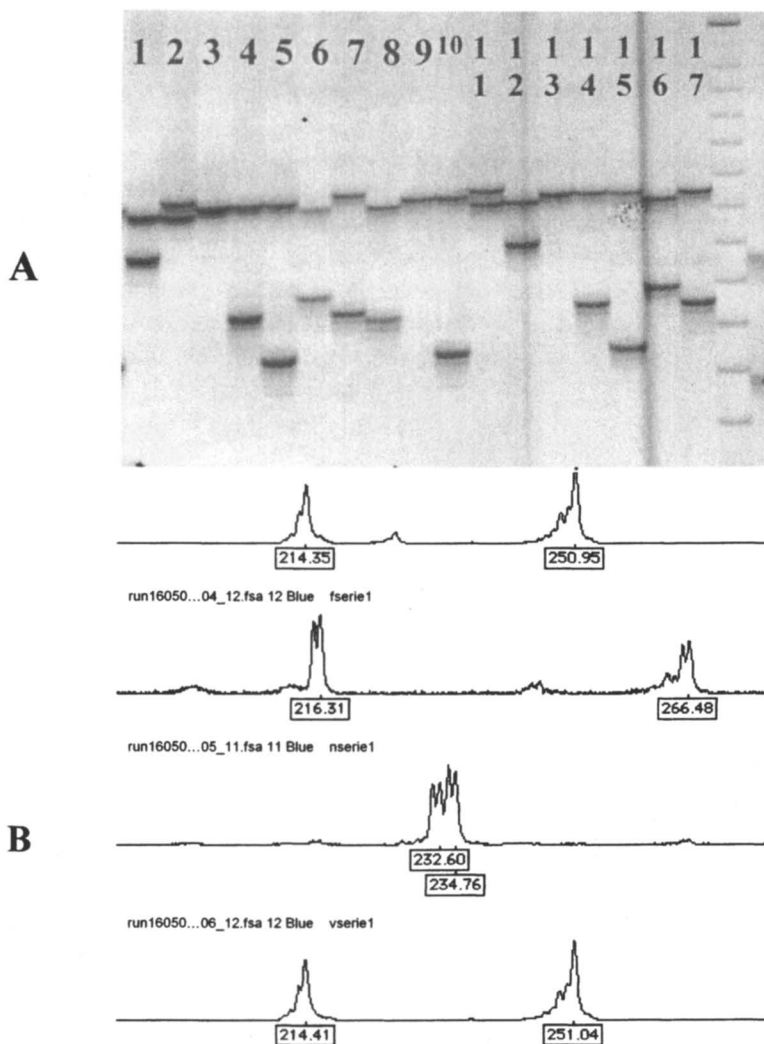


Figure 2. Comparison of the manual slab gel electrophoresis method and the automated capillary sequencing method. *A)* Silver stained slab gel electrophoresis of 10 rootstocks analyzed using SSR locus VVMD5. lane 1 & 12: R.S.B.1, lane 2 & 11: 44.53 Ma, lane 3 & 13: 1616C, lane 4 & 14: Viala, lane 5 & 15: 5C, lane 6 & 16: 125 AA, lane 7 & 17: 34 E.M., lane 7: 216-3 Cl, lane 8: 4010 Cl, lane 9: 196-7 Cl. *B)* Fluorescence detection coupled with capillary gelectrophoresis analysis of 5C, Chasselas, Cabernet-Sauvignon and 5BB using SSR locus VVMD28.

hairs (57) and of complex fluids such as blood and semen (11). The additional advantage of the RAPD method was that no prior knowledge of grape sequences was necessary, and several commercial companies developed commercial primers kits. Unfortunately, the RAPD method was demonstrated to have limited reproducibility (25, 58) and fragment selection was therefore necessary (15, 59). In addition, the resulting data was difficult to share among laboratories because of their limited reproducibility. The development of SCAR markers from RAPD fragment sequence data helped with reproducibility, but required some amount of sequencing. The SSR microsatellite markers quickly gained preference over RAPDs, despite the need for isolation of the fragments carrying SSRs and their sequencing. In addition to being more reproducible, the SSR markers were also more discriminating (2, 18, 48). The difficulties in their analysis with slab gel electrophoresis was greatly reduced when automatic sequencers were used, leading to the analysis of thousands of accessions around the world. At this point a second international effort was coordinated by E. Maul within the Genres 081 program (<http://www.genres.de/vitis>) to confirm the reproducibility of the technique and to promote the comparison of data among laboratories. The microsatellite markers proved to be highly reproducible, although some discrepancies were observed for heterozygous individuals with alleles differing by only one repeat (60). It was determined that successful comparison of data among laboratories required the use of a standard set of cultivars. This set of cultivars was defined for 6 loci (60) and will soon be defined at an additional 16 SSR markers (This et al., in preparation).

Despite the high degree of diversity, SSR markers are rarely capable of discriminating among clones. Efforts are now directed at using the AFLP method to distinguish clones. This method has the combined advantages of RAPD (no sequence information needed, high number of markers per analysis) and to some extent of SSR (reproducibility) and has proven to be very polymorphic. However, AFLPs have been found to produce variable results based on extraction procedures and the type of material analyzed (leaves versus wood), and therefore the technique needs to be fine-tuned by selecting the most reproducible fragments.

New marker systems are already in development. The SNP (single nucleotide polymorphism) marker system is already in use for human forensic analysis (61). Although little is known about their possible use in grape, SNPs have been shown to be present and numerous (62) and could lead to the development of markers capable of analyzing mutants and clones. Transposable elements such as retrotransposons were also discovered in grape (63-65). Markers based on the use of transposable elements such as S-SAP (Sequence-Specific Amplified Polymorphism) (66, 67), IRAP (Inter Retroelement Amplified Polymorphism) (41), or MSAP (Methyl-Sensitive Amplified Length

Polymorphism; 51) could also be very useful for clonal variation in the near future.

In conclusion, the SSR method is currently the method of choice for authentication of interspecific hybrids, rootstocks and cultivars, while SSR combined with AFLP is well adapted to the analysis of clones.

Analyzing DNA from Grape: Which DNA to Analyze?

In theory, every single cell of a grape plant possesses the same DNA, from the roots to the leaves. Therefore whatever the organ or tissue, it is always possible to identify a grape based on analysis of its DNA. The only limitation is the ease of DNA extraction and the availability of the plant material at a given time. Grape DNA extraction has thus been possible from leaves (18), wood (12, 59) and roots (This et al., unpublished), from berries and raisins (68), and from somatic embryogenic cells (69). It has also even been accomplished from archeological material (70). There are, however, a few exceptions to this rule. The seeds are issued from the fertilization of the egg cell (from the mother) by a pollen grain (from the father). The fingerprinting of seeds will therefore be different from that of the mother plant. In case of self pollination, the alleles will be the same as the parent's, but more loci will be homozygous in the seeds; in case of outcrosses, the seeds will only share half of the alleles with the mother plant. The other exceptions are chimeras. Chimerism has been shown to be frequent in grape (71-73). Often the leaves of chimeric plants will show a tri-allelic profile. But if the chimerism does not concern the whole plant, other organs or plant tissues (such as woody parts) will display different profiles.

The DNA analysis is usually performed on single plants, but it has been shown that DNA could be extracted from several plants (up to 10) and analyzed using SSR in order to analyze plant mixes (74, Laucou et al., in preparation).

Authentication by SSR: Power of the Markers and Diversity Level

Since the first analysis by Thomas et al. (75), the SSR microsatellite method has been successfully used for analysis of grape material. Several parameters have been used in order to describe the discriminating power of different markers. The number of alleles generated by a locus can be a first element. This parameter greatly varies among the different studies on grape and among the markers (3-19 alleles reported, Table III). Since this parameter is highly influenced by the sample size and the type of material analyzed, it is difficult to compare the different studies. Nevertheless, grape is very polymorphic and the different loci display large differences in allele numbers that can be explained

Table III. Diversity Revealed in Grape (*Vitis vinifera*) using SSR Markers: Parameters of the Diversity, Parameters of the Primers and Types of Identical Genotypes Detected

Reference	Parameters of the Diversity			Parameters of the markers			Identical genotypes
	Number Cultivar (Acc. ¹)	Number of SSR	Number Alleles (mean)	He ²	Number genotypes (PI ³)	D ⁴ or PIC ⁵ *(mean)	
85	366	8	5-19 (11.75)	0.771	244		Repetitions synonyms
105	69 (114)	8	7-9 (8)	0.71 - 0.95	56 (0.11 - 0.216)		Synonyms
106	20	8	3-6	-	20 (0.53-0.82)		
107	110	6	3 - 13	0.23 - 0.8	110 (0.11 - 0.66)		
108	74	9	4-10	0.70 - 0.82	74 (0.09-0.25)		
109	12	11	3-7	-	12 (0.12 - 0.3)		
110	50	11	7-9 (7.9)				Synonyms
111	49	11	5-9 (7)	0.67-0.84	36 (0.08-0.18)		Synonyms Clones
112	318 (176)	6	9-13 (11)	0.76-.09	163	0.89-0.95	Clones
113	43	8	2-8 (6)	0.39-0.95 (0.68)	43	36-78 *(60)	
114	164	9	4-13	0.71-0.851	164 (0.07-0.13)		
86	110	11	5-16 (9.2)	0.69-0.92	108	0.85-0.96 *(0.91)	Clones
115	80	9	5-9 (7)	0.71-0.98	55		Synonyms
75	80	7	-		79		Possible Errors

¹Accessions ²Observed Heterozygosity ³(77) ⁴(78) ⁵(76)

primarily by the nature (perfect, imperfect, compound) (75) and the length of the repeat (Laucou et al., in preparation). Another parameter, the polymorphism information content (PIC) has been proposed (76), but not widely used in grape. Several probabilities have also been used (Table III) such as the probability of identity (PI) (77) or the discrimination power (D) (78, 79). These parameters depend on population genetics rules and the corresponding assumptions about random mating in a grapevine population that may not be completely true. They therefore may not be well suited for grape, and a more simple, and conservative parameter such as the frequency of a genotype can be used ($\text{freq} = 1 / n$, where n is the number of typed cultivars in the database) (75).

The polymorphism revealed by SSRs is very different when analyzing diversity between *Vitis* species, between cultivars, or between clones. A very high polymorphism was detected among accessions of different *Vitis* species and among different rootstock varieties (79-81). A high variability is also detected among the cultivars of *Vitis vinifera* (Table III). Most of the studies listed in Table III show either a high to very high D (0.85-0.96) or a low to very low PI (0.08 - 0.2) among cultivars. The variability among clones is, on the contrary, highly reduced for SSR, which makes these markers very useful for cultivar authentication only. However, in some cases, when using a very high number of SSR loci, a low level of diversity was revealed between clones of several cultivars (71-73, 82-84) allowing the identification of individual clones.

A very important question is the minimal number of SSR markers to use in order to insure authentication with very high fidelity. In the case of different rootstocks and grape species, due to the high diversity, 4 to 5 SSR markers as proposed by Lamboy and Alpha (79) are probably sufficient. In the case of *Vitis vinifera* cultivars, it is more difficult to answer this question. The studies listed in Table III (which do not represent an exhaustive list, but a selection of the main studies) analyzed 12 to 366 individuals with 6 to 11 loci. A high number of genotypes were obtained, but in many cases, a few samples could not be differentiated. The authors concluded that the undifferentiated samples represented clones of the same cultivars or synonyms. Such samples were either already suspected of being synonyms or clones based on analysis of morphological traits, or they were completely unknown clones. From these studies, one would be tempted to suggest that 6 to 10 loci are sufficient, since they were sufficient to distinguish up to 244 cultivars (85). Siret (86) showed that analysis of 109 cultivars of diverse origin was possible with 3 SSR loci. Nevertheless, although these numbers may be sufficient for confirmation of identity, they may still be limited for "blind" analysis. In fact, Filippetti et al. (87), analyzing plants obtained from the self-pollination of the Sangiovese cultivar, found that two individuals issued from two different seeds shared identical profiles for at least 10 SSR loci.

Since the published studies used different loci and different sets of cultivars, it is difficult to estimate the number of markers that would be necessary for accurate authentication. It is therefore still difficult to determine the minimal set of SSR markers necessary for blind analysis. The loci should surely be selected on the basis of the different parameters described earlier, but also on their position on the grape genome. Blouin et al. (88) stressed that the efficiency of the markers increases with well-dispersed loci. The ease of scoring should also be fundamental in the choice of loci. This et al. (60) used 6 very informative loci, some of which should be universally tested. We have initiated the analysis of the Vassal grape collection (about 2250 different cultivars) using 20 well-distributed SSR markers. The analysis of this collection, including many of the most important cultivars worldwide, as well as a fairly large selection of old local varieties will soon be completed (Laucou et al., in preparation). This study will allow the definition of a minimal set of SSR markers capable of reliably identifying cultivars (This et al., in preparation).

If the molecular markers can easily be used for the authentication of plant material from a very large range of organs and tissues (excluding seeds), is it possible to use these markers to authenticate processed grape products such as juice and wine? This process requires two different analyses: authentication of the cultivar(s), and estimation of the quantity of the different cultivars in a mix. Can DNA analysis and genotyping enable such characterization ?

Application to the Analysis of Complex Solutions: What can Wine DNA Reveal?

In order to estimate the possibility of determining the varietal composition of juice and wine by DNA analysis, one needs to understand the origin of DNA present in these products and its evolution. DNA in the juice originates mainly from the berries and can be extracted from the dry matter still present in solution and the authentication is possible (33, 89). In the wine, the DNA also originates from the grape berries, but during fermentation, many microorganisms develop (yeast, bacteria, fungus) and they of course contain DNA. The wine is thus a mix of different types of DNA. During the fermentation, the quantity of dry matter decreases, reducing the quantity of grape DNA capable of being extracted (33). At the end of the fermentation, when the wine is taken from the barrels and is further treated, the DNA is only present in solution, and is very dilute. During fermentation and storage of the wine, catalytic enzymes as well as the acidic conditions of the wine (pH = 2-4) will degrade the DNA. Thus, the analysis of DNA in wine will require specific markers that work with small fragments of DNA. SSR markers do correspond to this description. Since the DNA is very dilute however, extraction procedures will have to concentrate DNA and prevent

a range of potential inhibitors (e.g., polyphenols, polysaccharides or RNA) from preventing SSR amplification (90, 91).

These limitations make the analysis of DNA from wine very difficult. It has been possible from experimental or unfiltered and unprocessed wines (33), but it is not yet consistently possible from commercial wines. There has been some success with characterizing mixtures of cultivars in wine using SSR markers (92), but these analyses have only been performed on experimental wines. New developments in DNA extraction and in molecular markers will therefore be necessary in order to enable the authentication of cultivars in commercial wines.

Conclusions

SSR markers are the most popular markers for the authentication of species and cultivars from any grape tissue. Comparison of data is easy, but not straightforward, and may require the need of some coding and reference standard cultivars. There are possible discrepancies in the amplification and the analysis that can also be observed. Coupled with the possibility of chimeras, the authentication of grape cultivars should thus be made with precautions. In addition, a sufficient number of SSR markers are necessary. A study is underway to confirm the minimal number of SSR markers required for authentication with greatest accuracy.

When associated with AFLP markers, SSR markers are also useful for characterization of clones, although a reliable method for identifying clones in many cultivar backgrounds is not yet complete. New developments in marker methodology should enable the identification and authentication of clones in the future. Finally, when SSR marker systems are applied to grapes processed as experimental juice and wine, they have been able to authenticate the grape cultivars in the wines and, to a more limited extent, quantify the amount of various cultivars in a blended wine. The analysis of commercial wine is not possible, and new development in DNA extraction methods and quantitative markers will be needed to achieve this goal.

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Chapter 15

Anthocyanin, Flavonol, and Shikimic Acid Profiles as a Tool to Verify Varietal Authenticity in Red Wines Produced in Chile

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Chilean wine exports increased from 52 to 835 millions US-dollars between 1990 and 2004. They mainly consist of the red varieties Cabernet Sauvignon, Merlot and Carmenère. For sustainable long term growth, it is essential to develop a reliable system to verify product authenticity. For varietal authenticity in red wine, various authors proposed the use of the ratio between acetylated and coumaroylated anthocyanins (Rac/cum). In the present paper, the anthocyanin, flavonol, and shikimic acid profiles were determined in wines produced in Chile during the vintages 2001-2004, in order to differentiate between wine varieties. The results of principal component analysis of anthocyanins show that it is possible to differentiate between the three grape varieties using only their anthocyanin profiles. This is confirmed by linear discriminant analysis of these compounds, which classified well a total of 95.8% of the investigated wines. Only by the Rac/cum ratio, it is not possible to differentiate between Merlot and Carmenère, however, this ratio tends to discriminate between Cabernet Sauvignon and the other two varieties. The concentration of shikimic acid improves the differentiation between Cabernet Sauvignon and Merlot or Carmenère, but not between the latter two varieties. With respect to flavonols, our results show that the ratio of total quercetin/ total myricetin (TQ/TM) in combination with peonidin-3-glucoside in a simple ratio or

with all anthocyanins determined in a model improves the differentiation between Carmenère and Merlot. The high error of classification in Merlot is in part attributed to the fact that in Chilean vineyards this variety was previously confused with Carmenère.

Chilean wine exports increased from 52 to 835 million US-dollars between 1990 and 2004. They mainly consist of the red varieties Cabernet Sauvignon, Merlot and Carmenère. For sustainable long-term growth, it is essential to develop a reliable system to verify product authenticity. In this sense, various authors have proposed to differentiate between red wine varieties using their anthocyanin profiles (1-7). The validity of this approach was questioned a few years ago by Burns and coworkers (8), but these authors did not adequately consider the marginal conditions of the original method (compounds, age of wine, detection mode, sample volume).

Holbach *et al.* (4) proposed additionally the combination of anthocyanin profiles with shikimic acid concentrations to differentiate between red wine varieties. Fischerleitner (9) concluded that only Cabernet Sauvignon can be identified through its shikimic acid content alone, which for wines produced in Austria is far beyond that in wines of other varieties. However, most authors only considered simple relations between these compounds. The method approved by the OIV in 2003 is also based on this principle (10). More complex methods for classification purposes, based on anthocyanin profiles, but including statistical models, have been proposed by Berente (3), Otteneder (5), de Villiers (11), and von Baer (12). For wines from 5 grape varieties produced in South Africa, de Villiers (11) recently reported an attempt to differentiate between different cultivars according to the anthocyanin content using stepwise forward linear discriminant analysis (LDA). On the other hand, Aleixandre (13) proposed to classify young red varietal wines produced in the Valencian community (Spain) by discriminant analysis with 33 variables: 9 conventional parameters, 10 alcohols and polyols and 14 esters. Considering only 11 variables, it was possible to differentiate 100 % of the samples.

A completely different approach was published very recently by Beltrán (14) to differentiate between Chilean Cabernet Sauvignon, Merlot and Carmenère wines. They processed the whole polyphenol chromatogram, using a genetic algorithm to select 37 points of the original chromatogram to differentiate between these three red varieties.

In the present paper, the anthocyanin, flavonol and shikimic acid profiles were determined by HPLC in wines produced in Chile during the vintages 2001 - 2004, in order to differentiate between the three main red wine varieties produced for export in Chile. For multivariate data analysis the principal components analysis was applied. The simple ratios were evaluated using categorized graphs and classification between grape varieties was made using

linear discriminant analysis. Discriminant analysis was applied to the following chemical markers: proportions of nine anthocyanins, anthocyanins in combination with shikimic acid concentration and proportions of flavonols. In case of anthocyanins, to make the results more comparable with that of the OIV-Method (10), only the proportions of the nine anthocyanins established in that method, taking their sum as 100 %, were considered. We are not taking into account other minor anthocyanines, like vitisin A, which is present in wines of the studied varieties, but was found to be much more stable than other monomeric anthocyanins considered in the OIV-Method (15).

Material and Methods

Wine Samples

All wine samples came directly from wineries located at the valleys of Casablanca, Maipo, Rapel, Colchagua, Curicó, Maule, Itata and Bío-Bío in Chile. They corresponded to the vintages 2001, 2002, 2003, and 2004. Vinification was made at production scale and samples were taken after malolactic fermentation and before blending. No commercially available bottled wine samples were included in the study. The grape variety for each sample was declared by the producer and considered correct for the purposes of this study. Samples are summarized in Table I.

Table I. Sample size per vintage year and grape variety

	2001	2002	2003	2004	Total
Carmenère	8	24	36	28	96
Merlot	9	29	22	16	76
Cabernet Sauvignon	20	70	37	104	231
Total	37	123	95	148	403

Analytical Methods

Before chromatographic analysis, all wine samples were passed through a GV Durapore filter (0.22 μm pore size, 13 mm diameter) (Millipore, USA). Anthocyanin determination by reversed phase HPLC is based on the method described by Holbach *et al.* (2), Otteneder *et al.* (5), and OIV (10) with minor modifications. A Lichrocart 4-4 Lichrospher 100 RP-18 (5 μm) was used as pre-column, and a Lichrocart Purospher RP-18e 5 μm as analytical column. Both

columns are from Merck, and they were operated at a temperature of 50 °C and a flow rate of 1.0 mL min⁻¹. The chromatographic system consisted of a quaternary LC-10ADVP pump, a FCV-10ALVP elution unit, a DGU-14 degassing unit, a SIL-10ADVP autoinjector, a CTO-10AVP column oven, a SPD-M10AVP diode array UV/VIS detector, a SCL-10AVP controller, and a CLASS-VP data system, all from Shimadzu Corporation, Japan. The sample volume was 50 µL and detection at 518 nm. Only the 9 peaks defined by the method were considered.

We determined the concentration of shikimic acid and other organic acids present in wine by a combination of reversed phase and ion exclusion chromatography in series, as described by Holbach *et al.* (4) and by OIV (16), but at a mobile phase flow rate of 0.5 mL min⁻¹. Both columns were provided by Institut Heidger, Kesten, Germany. The chromatographic system consisted of a Perkin Elmer 250 binary pump, a column oven at 70 °C, a Shimadzu SPD-10AV UV VIS detector set at 210 nm and Hewlett Packard Chem Station data system. Sample volume: 6 µL.

Flavonols were determined by HPLC based on the methodology of McDonald *et al.* (17) with minor modifications. The separation was carried out on a reversed phase C-18 column (Shimpack VP ODS, 150 x 4.6 mm, Shimadzu) at 40 °C, with an acetonitrile/trifluoroacetic acid gradient at pH 2.5 as mobile phase at 0.7 mL min⁻¹ and detection at 365 nm. Free flavonols were determined directly and the conjugated forms after acid hydrolysis of the wine samples (90 °C). Table II show a list of all compounds determined in wine samples.

Table II. Measured parameters

<i>N</i> ^o	<i>Parameter</i>	<i>Abbreviation</i>
1	% delphinidin-3-glucoside	Dp
2	% cyanidin-3-glucoside	Cy
3	% petunidin-3-glucoside	Pt
4	% peonidin-3-glucoside	Pe
5	% malvidin-3-glucoside	Mv
6	% peonidin-3-acetylglucoside	Peac
7	% malvidin-3-acetylglucoside	Mvac
8	% peonidin-3-coumaroylglucoside	Pecu
9	% malvidin-3-coumaroylglucoside	Mvcu
10	Shikimic acid concentration [g L ⁻¹]	Shi
11	Total myricetin [mg L ⁻¹]	TM
12	Total quercetin [mg L ⁻¹]	TQ

Statistic Methods

The statistic analyses included categorized scatterplots to find relations among chemical markers and establish relevant ratios. For multivariate exploratory analysis, we used principal components, and for classification purposes, we employed linear discriminant analysis (18). Statistical analyses were performed with the S-Plus 6.1 (Insightful Corp., USA) and STATISTICA 6.0 (StatSoft, Tulsa, OK, USA) software packages.

Results and discussion

For the harvests 2001 – 2004 the figures obtained by the Rac/cum ratio of OIV (10) for a total of 403 red wine samples were similar to those reported and discussed previously by the authors for the harvests 2001-2003 (12).

The results of principal components analysis for the harvests 2001 – 2004 are presented in Figure 1 and 2. Figure 1 shows the factor loadings for the

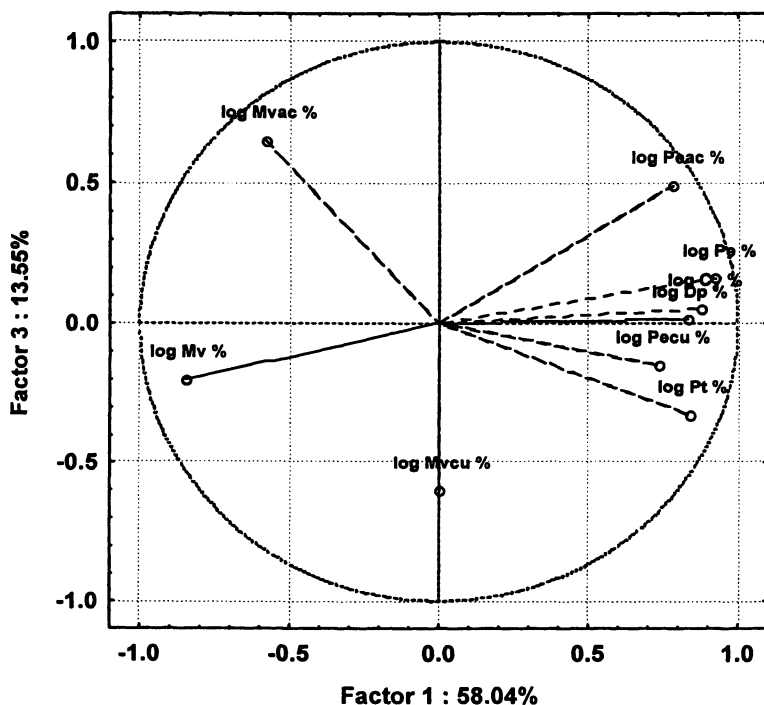


Figure 1. Loadings of principal components analysis of the proportions of nine anthocyanins.

logarithm of proportions of the nine anthocyanins listed in Table 2. Factor 1 explains 58 % of the variance and factor 3 explains 13.5 %. Factor 1 represents a contrast among Mv, Mvac and Pe, Peac, Pt, Pecu, Dp, Cy, factor 3 contrasts acetylated with coumaroylated anthocyanins. Factor scores (Figure 2) clearly indicated the formation of three groups that correspond to the three grape varieties. Figure 2 shows that factor 3 separates Carmenère from Cabernet Sauvignon. Carmenère is characterized by high proportions of Mv_{cu} and Cabernet Sauvignon by high proportions of Mvac. Merlot presents greater proportions of Pe, Peac, Pecu, Dp, Pt and Cy.

The relation between shikimic acid concentrations and the Rac/cum ratio is presented in Figure 3. Both tend to discriminate between Cabernet Sauvignon and the other two varieties, but not between Merlot and Carmenère. This separation is indicated by the dotted lines.

In case of flavonols, the discriminating power of several relations between them were tested, concluding that the relation between the ratio of total quercetin/total myricetin (TQ/TM) together with the concentration of peonidin-3-glucoside could be effective parameters to differentiate between Carmenère and Merlot wines (Figure 4).

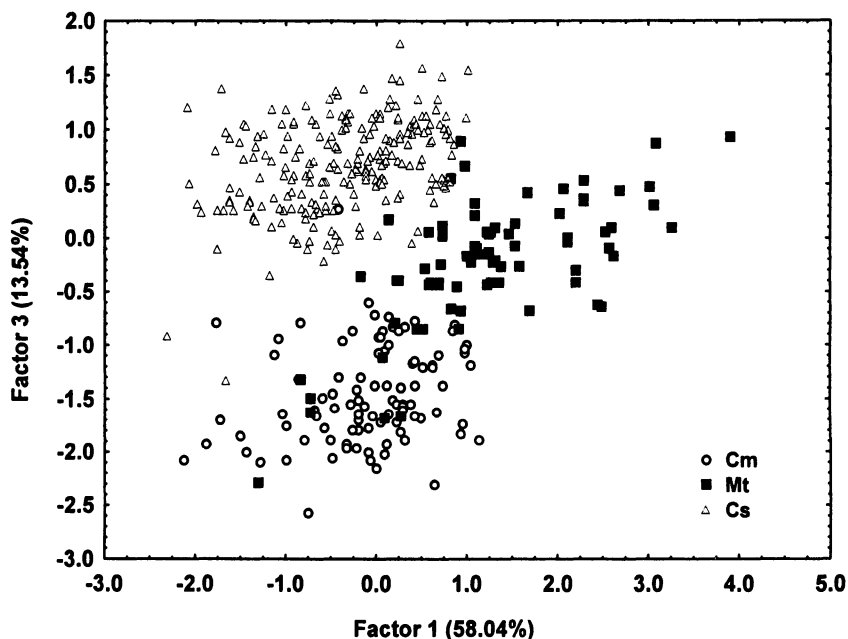


Figure 2. Scores of principal components analysis (Cs = Cab. Sauvignon, Mt = Merlot, Cm = Carmenère)

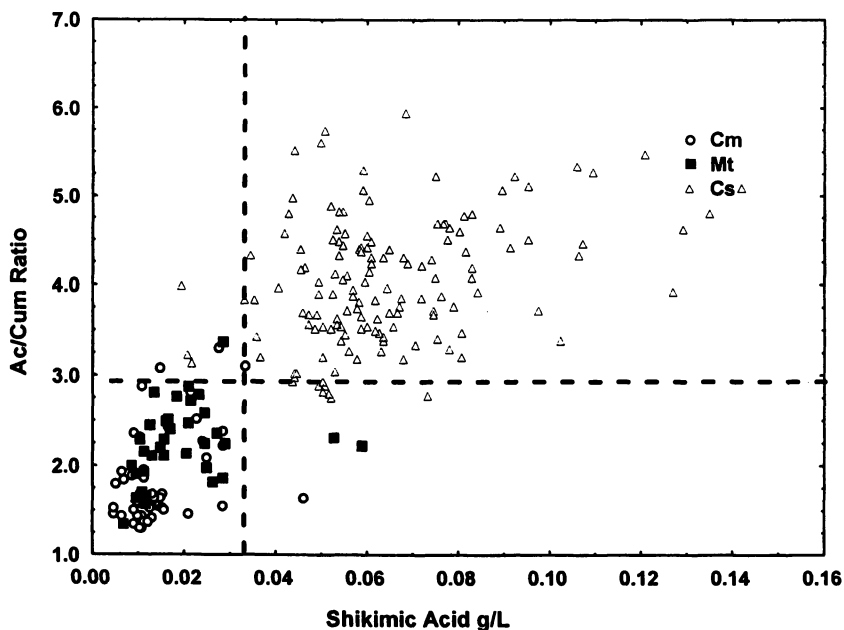


Figure 3. Dispersion between *Rac/cum* and shikimic acid concentration for *Carmenère* (Cm), *Merlot* (Mt) and *Cabernet Sauvignon* (Cs), vintages 2002-2004, Chile ($n = 227$).

To prove the discriminating power of anthocyanins (Table III), anthocyanins and shikimic acid concentrations (Table IV), anthocyanins and TQ/TM (Table V), and all parameters together (Table VI) linear discriminant analyses were performed. Although the sample sizes for anthocyanins ($n = 403$), anthocyanins and shikimic acid ($n = 227$), anthocyanins and flavonols ($n = 275$), and all parameters together ($n = 150$) was different due to operative limitations in analytical systems, it was considered relevant to evaluate and discuss the incidence of the different parameters, besides anthocyanins, in the classification system. Table III show that the major error is observed for Merlot, whereas *Carmenère* and *Cabernet Sauvignon* presented very low errors (1 to 2%). When shikimic acid concentrations are incorporated in the model (Table IV), the error of *Cabernet Sauvignon* is reduced to zero and total error decreases in one point.

Other authors (14) reported very recently a total classification error of 5.81% for the same three grape varieties in Chile, but using a completely different approach to process the whole chromatogram, from which they select 37 data points, instead of certain specific peaks.

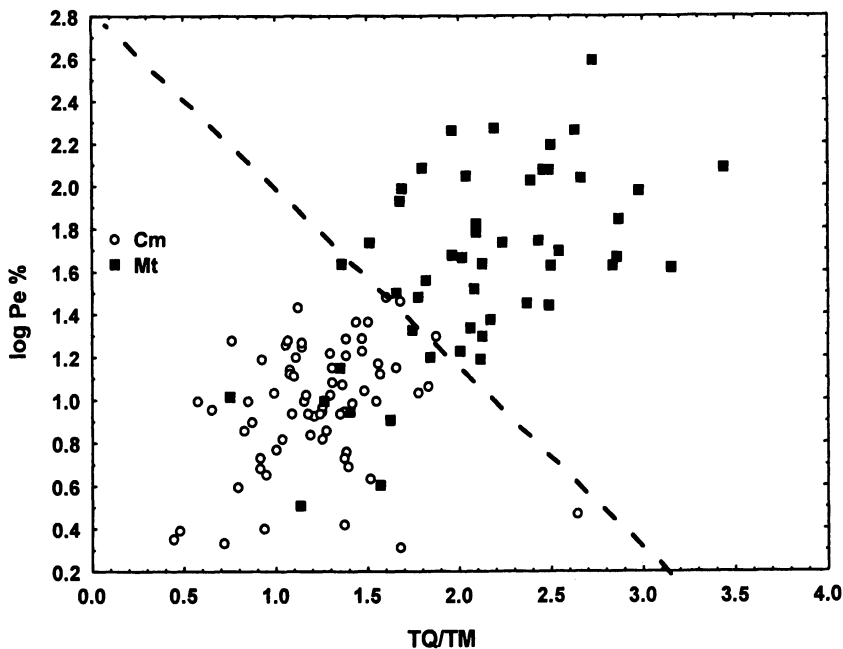


Figure 4. Relation between the ratio Total Quercetin/Total Myricetin (TQ/TM) and logarithm of peonidin-3-glucoside proportions in Merlot (Mt), Carmenère (Cm) and Cabernet Sauvignon (Cs), vintages 2002 - 2004, Chile ($n = 275$).

Table III. Linear discriminant analysis of nine anthocyanins ($n=403$)

Variety	Carmenère	Cabernet Sauvignon	Merlot	Error
Carmenère	94	1	1	2.1 %
C. Sauvignon	1	228	2	1.3 %
Merlot	12	0	64	15.8 %
Total Error				4.2 %

Table IV. Linear discriminant analysis of the nine anthocyanins and shikimic acid ($n= 227$)

Variety	Carmenère	Cabernet Sauvignon	Merlot	Error
Carmenère	45	1	1	4.2 %
C. Sauvignon	0	144	0	0.0 %
Merlot	5	0	31	13.9 %
Total Error				3.1 %

If in the model the ratio of TQ/TM is combined with the proportion of the nine anthocyanines, the error of Merlot and Carmenère decreases (Table V). When all chemical markers are combined in a model the total error decreases, however the error for Carmenère and especially for Merlot increases.

Table V. Linear discriminant analysis of nine anthocyanins plus TQ/TM (n = 275)

<i>Variety</i>	<i>Carmenère</i>	<i>Cabernet Sauvignon</i>	<i>Merlot</i>	<i>Error</i>
Carmenère	68	1	0	1.4 %
C. Sauvignon	0	155	1	0.6 %
Merlot	6	0	44	12.0 %
Total Error				2.9 %

Table VI. Linear discriminant analysis of 9 anthocyanins plus TQ/TM and shikimic acid concentrations (n = 150)

<i>Variety</i>	<i>Carmenère</i>	<i>Cabernet Sauvignon</i>	<i>Merlot</i>	<i>Error</i>
Carmenère	28	1	0	3.4 %
C. Sauvignon	0	101	0	0.0 %
Merlot	3	0	17	15.0 %
Total Error				2.7 %

Whereas the error of Cabernet Sauvignon and Carmenère varies from 0 to 4 % in all models, the errors of Merlot reach up to 15 %. This result can be explained as follows: Some years ago, Carmenère, which in other countries disappeared due to phylloxera, was rediscovered in Chile. Formerly, all vineyards planted with this grape variety in Chile were declared as Merlot. Hinrichsen (19) using SSR DNA markers to confirm the varietal identity, found that from a total of 93 vines of five Chilean vineyards, originally planted as Merlot, four vines matched Carmenère. This leads to the conclusion that at this time, those vineyards declared as Carmenère are correctly identified with a high probability, but a certain percentage of vineyards declared as Merlot, still correspond to Carmenère. This confusion contributes significantly to the high error rate for samples declared to be Merlot.

To illustrate the behavior of misclassification error excluding Merlot, a discriminate analysis was made including all chemical markers listed in Table II. The results are presented in Table VII. Now the total error does not surpass 1%.

Table VII. Linear discriminant analysis of anthocyanins plus TQ/TM plus shikimic acid concentrations excluding Merlot (n = 130)

<i>Variety</i>	<i>Carmenère</i>	<i>Cabernet Sauvignon</i>	<i>Error</i>
Carmenère	28	1	3.4 %
Cabernet Sauvignon	0	101	0.0 %
Total Error			0.8 %

Independent of the chemical markers used to differentiate between grape varieties, it is important to consider that the border conditions of the analytical method should be respected rigorously, to avoid mistakes like those of other authors (8), who used other chromatographic conditions, detection mode and did not properly consider the age of the wine. It is highly recommended that at the time of analysis, the wine has stabilized after malolactic fermentation and that is not older than three years. On the other hand, the particular processing technology, i.e. the use of some pectinolytic enzymes and the termovinification process, both pointed out by Fischer *et al.* (20) in this symposium, as well as the interannual variations must be considered.

Conclusions

Principal components analysis show that it is possible to differentiate between the three grape varieties under investigation using only their anthocyanin profiles.

The simple Rac/cum ratio tends to discriminate between Cabernet Sauvignon and Carmenère or Merlot, differentiation which is improved by the combination with the concentration of shikimic acid.

The combination of the proportions of the nine monomeric anthocyanins with the TQ/TM ratio in a simple relation or in a model improves the differentiation between Carmenère and Merlot.

Some vineyards declared in Chile as Merlot may still correspond to Carmenère. This increase the misclassification error of Merlot and explains the low performance reached by the linear discriminant model for this variety.

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Chapter 16

Red Wine Authenticity: Impact of Technology on Anthocyanin Composition

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Assessment of authenticity of red wine varieties has been proposed on the ratio of acylated/non acylated and acetylated/coumaroylated anthocyanins. However, there is a lack of knowledge regarding modification of these ratios due to technological factors such as enzyme treatment, thermovinification or duration of skin contact. For the vintage 2001 and 2002, red wines were made from authentic grapes of the cultivars Dornfelder and Portugieser by thermovinification and fermentation on the skins. Increasing the temperature during thermovinification from 60 to 87°C and extending skin contact of the cooled mash at 40°C from 0 to 12 hours, enhanced the ratio of acetylated/coumaroylated anthocyanins. Prolonging skin contact during fermentation on the skins from 6 to 18 days induced higher ratios of acetylated/coumaroylated anthocyanins. Assessment of several commercial pectinases revealed significant acetyl- and/or coumaroyl esterase side activities. Despite the fact that only authentic varietal grapes were used for experimental wine making and applying only legal and state of the art enological treatments, principal component analysis of the experimental wines of Portugieser variety revealed a substantial dispersion. Due to the impact of technology some of the Portugieser and Dornfelder wines were outside the postulated confidence intervals of the acylated/nonacylated and acetylated/coumaroylated ratios which define authenticity. In conclusion, the modifying impact of red wine technology should gain more consideration in order to protect producers and wine trade against false alarms.

World wide, red wine gained market share due to a broad change in consumer preference. Anthocyanins contribute largely to the color of red wine and as a consequence to its quality. The five anthocyanidins found in grapes and wines are modified by glycosidation, which can be esterified with aliphatic acids such as acetic acid and phenolcarbonic acids such as coumaric or caffeic acid, yielding a wide range of compounds (10). The anthocyanin composition has been used by several authors as a chemotaxonomic marker to differentiate grape varieties at the level of non fermented grape samples (6, 14, 20) and at the level of finished wines (3, 4, 5, 7, 9, 12, 19). Analytical assessment of authenticity of red wine varieties has been proposed using the sum of acylated anthocyanins, the ratio of acetylated/coumaroylated anthocyanins and the concentration of shikimic acid (12). The official OIV method applies a HPLC-UV/VIS assay which is based on the five anthocyanin-3-glycosides present in wine and the acetylated and coumaroylated malvidin-3-glucoside and peonidin-3-glucoside, respectively (17). Due to the lack of reference compounds, the peak areas detected at 518 nm of those nine anthocyanins are added and their sum is set as 100%. Sums and ratios are calculated by using percentage figures of the different anthocyanin groups. However, there is a lack of knowledge regarding modification of these ratios due to technological factors such as enzyme treatment, yeast selection, thermovinification, and duration of skin contact.

Pectolytic enzymes are widely used during winemaking and are essential during thermovinification, because the native grape enzymes and those expressed by microorganisms on the grape are inactivated by the application of a temperature of 87°C for several minutes. Further on, it has been shown (23) that color extraction and stabilization could be enhanced using pectolytic enzymes. However, technical enzymes offer an array of side activities such as B-glycosidase and esterases, having an impact on the anthocyanin composition (7). For example increased β -glycosidase activities were correlated with lower amounts of monomeric anthocyanins and stronger polymerization (22).

It was the objective of this research project to systematically study the impact of selected technological factors on the anthocyanin composition, especially the sum of acylated anthocyanins (Σ acyl. anth.) and the ratio of acetylated/coumaroylated anthocyanins (Rac/cum). In the case that red wine technology indeed modifies the anthocyanin composition, it was important to examine whether technological factors are within or outside the published 9500 confidence range of both authentication parameters (1, 12, 16).

Experimental

Experimental wine making: In 2001 and 2002, red wines were made from authentic grapes of the cultivars Dornfelder and Portugieser by

thermovinification and fermentation on the skins. The grapes were harvested from specialists who were familiar with the ampelographic differences between the grape varieties, ensuring 100% authentic grape material. During thermovinification, the destemmed and crushed grapes were heated in a two step process to 60°C or 87°C. After maintaining the end temperature for two minutes, the mash was cooled to 40°C and extended skin contact was applied at the constant temperature of 40°C for 0 to 12 hours. The grapes were pressed after 0, 4 or 12 hours and the juices were fermented at 25°C using the same oenoferm rouge yeast strain (Erbslöh, Geisenheim, Germany) and alpha strain of malolactic bacteria (Lallemand, Montreal, Canada). SO₂ was added 4 weeks after malolactic fermentation was completed. Prior bottling, the wines were cross flow filtered (tangential flow). The same grapes were also subjected to fermentation on the skins in 220 Liter containers at constant temperature of 25°C. During fermentation (3-5 days), the cap was manually disintegrated and submerged three times a day. When fermentation had finished this punch down procedure was reduced to two times a day. Aliquots of the homogenized mash was removed from the containers after 6, 10, 14, or 18 days of fermentation/maceration. The same wine making was applied as for thermovinified red wines.

Analysis of anthocyanins by HPLC-DAD: Anthocyanin composition was measured at 90 min intervals during the holding time following thermovinification. For maceration on the skin trials, anthocyanins were measured once a day and every second day when fermentation had finished. Anthocyanins were detected by a HPLC method employing UV/VIS diode array detection (Jasco 935), a gradient HPLC pump (Jasco 975) and a Synergi Max RP (Phenomenex) column at 50°C. At a constant flow rate of 0.5 ml min⁻¹ solvent A consisted of 10 mmol/l aqueous KH₂PO₄, acidified with H₃PO₄ to pH 1.6 and gradient B of 50% of solvent A + 50% vol. acetonitrile following the gradient program listed in Table I. The Synergi Max RP column was applied because of its stability at very low pH (1.5). Sensitivity is enhanced at very low pH, because the majority of anthocyanins are present in their colored flavylium cation form. According to Wulf and Nagel (24), at pH 1.5 96% of the anthocyanins are in their colored form, while at pH 2.5 only 67% are colored.

Table I. Specification for HPLC-analysis and gradient program

<i>t</i> [min]	<i>solvent A</i>	<i>solvent B</i>
0	100%	0%
5	100%	0%
10	83%	17%
60	45%	55%
65	100%	0%

Identification of the non-acylated anthocyanins followed the typical elution order based on different degree of hydroxylation and methoxylation at the phenol B ring and their impact on the absorption maxima as displayed in Table II. Identification of acylated anthocyanins were based on the analysis of heated red Portugieser juice, where neither polymeric substances nor reaction products between anthocyanins and fermentation products such as vitisin A were present. For the identification of acetylated anthocyanins the commercial pectinase Trenolin rouge DF (Erbslöh, Geisenheim, Germany) was applied, which exhibits a strong esterase side activity and cleaves all acetylated anthocyanins. Coumaroylated anthocyanins could be identified due to a specific shoulder at 310 nm caused by their coumaric acid moiety. Besides the nine anthocyanins used for authentication assessment, the acylated derivatives of delphinidin-3-glucoside and petunidin-3-glucoside were routinely identified. Using a RP column of lower separation power than the Max RP column (2, 11, 16), the coumaroylated delphinidin-3-glucoside and the acetylated malvidin-3-glucoside may coelute as well as pinotin A and the coumaroylated malvidin-3-glucoside (21).

Table II. Order of elution during RP-HPLC of non-acylated anthocyanins and their respective absorption maximas

<i>elution order</i>	<i>anthocyanin</i>	<i>R¹</i>	<i>R²</i>	<i>R³</i>	<i>absorption maximum [nm]</i>
1	Delphinidin -3- glucoside	OH	OH	OH	523
2	Cyanidin-3-glucoside	OH	OH	H	515
3	Petunidin-3- glucoside	OCH ₃	OH	OH	527
4	Peonidin-3- glucoside	OCH ₃	OH	H	519
5	Malvidin-3- glucoside	OCH ₃	OH	OCH ₃	527

Results and Discussion

Fermentation on the skins

Fermenting Dornfelder grapes on their skins during an 18 day time period revealed for malvidin-3 -glucoside the expected extraction curve, characterized by a fast increase, a maximum after 4 days, a decrease, presumably due to polymerization of the anthocyanins and finally a stable level. While the acetylated malvidin-3-glucoside behaved similar to the non-acylated malvidin-3-

glucoside, the concentration of the coumaroylated malvidin-3-glucoside showed a less pronounced increase during the first 2 days followed by a slow decrease to a rather low stable level (Figure 1).

This coincides with reports that coumaroylated anthocyanins were stronger diminished during wine making than their acetylated counterparts (13). It can be speculated that coumaroylated anthocyanins were extracted at a lower rate or that monomers were faster removed by enhanced polymerization. Another explanation could be oxidation of the coumaric acid moiety. As a consequence of the weaker enrichment of coumaroylated anthocyanins, the ratio of acetylated/coumaroylated anthocyanins increased from the 2nd day until the 12th day of fermentation from 1 to 3.6 (Figure 2). The sum of acylated anthocyanins decreased during the first 5 days of fermentation from 21% to 11%, presumably due to a cleavage of the ester bond. Thus, both authentication criteria changed during the winemaking process considerably.

The fermentation and maceration regime during red wine making is varied based on stylistic and economic considerations of the winemaker. In order to produce fruity red wines with less tannins versus wines with more structure and tannins, the skin contact of Dornfelder is varied in wineries between 2 to 14 days. Based on the reported results, different maceration periods not only alter sensory properties, but also both authentication parameters substantially. Using

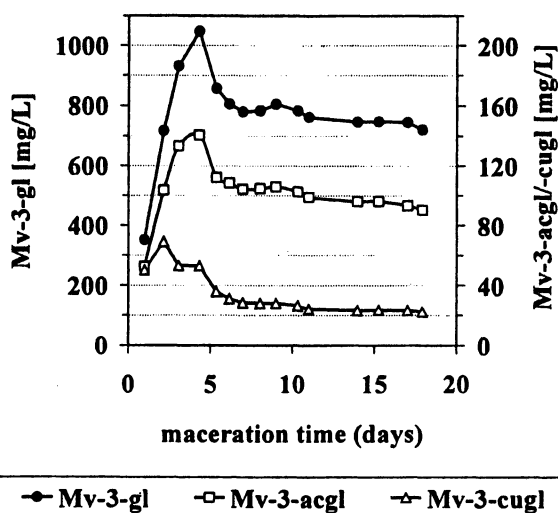


Figure 1. Change of concentration of malvidin-3-glucoside derivatives during fermentation on the skins of 2001 Dornfelder red wine

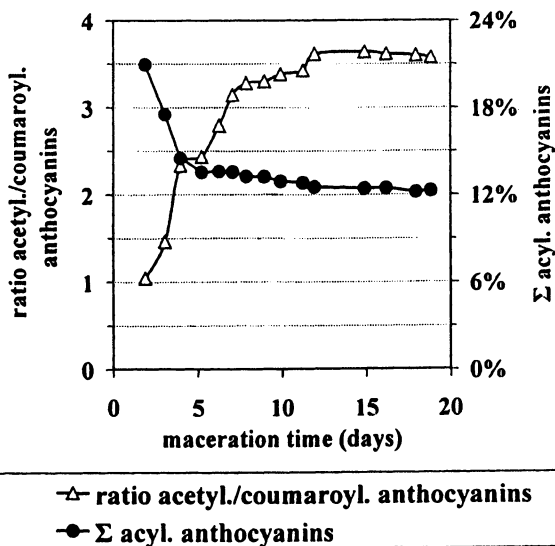


Figure 2. Alteration of acetylated/coumaroylated anthocyanin ratio and sum of acylated anthocyanins during fermentation on the skins of 2001 Dornfelder red wine

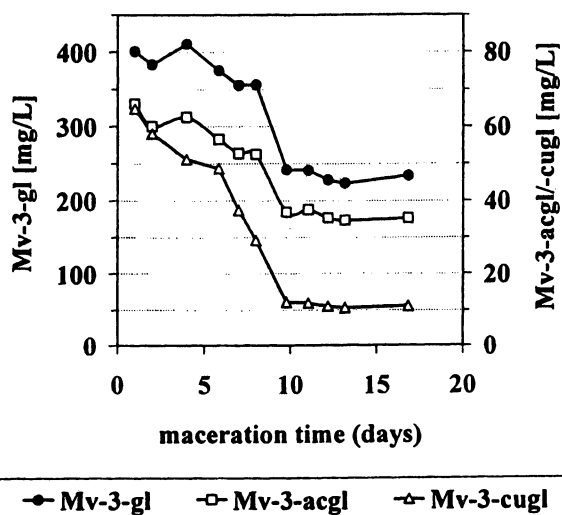


Figure 3. Change of concentration of malvidin-3-glucoside derivatives during fermentation on the skins of 2001 Portugieser red wine

the same grape material, a short skin contact of 3 days produced a press wine with 18% acylated anthocyanins and a Rac/cum of 1.45, while an extended skin contact of 10 days yielded a press wine with 12.9% acylated anthocyanins and a Rac/cum as high as 3.3.

Maceration on the skins of Portugieser grapes yielded a similar pattern as for Dornfelder: Thus concentration of coumaroylated malvidin-3-glucoside decreased faster than the acetylated or non-acetylated malvidin-3-glucoside (Figure 3). It took 10 days, until both authentication parameters reached a stable level. In consequence, Rac/cum increased during 10 days most significantly from 1 to 3 and acylated anthocyanins decreased from 3.7 to 2.75 (Figure 4).

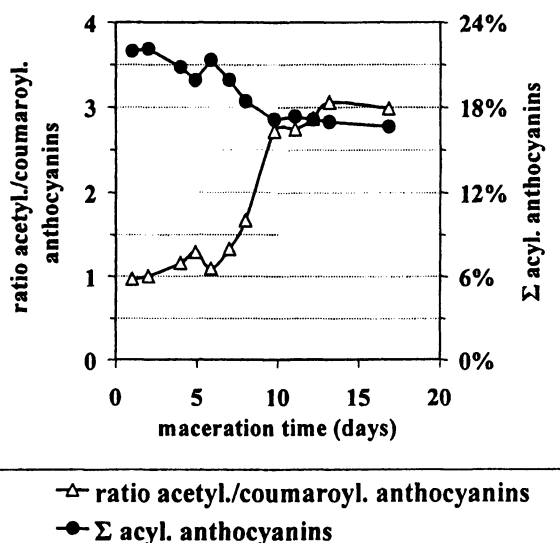


Figure 4. Alteration of acetylated/coumaroylated anthocyanin ratio and sum of acylated anthocyanins during fermentation on the skins of 2001 Portugieser red wine

Examining the authentication parameters in the bottled wines of both varieties, the percentage of acylated anthocyanins were only slightly modified by different skin contact times, but the Rac/cum varied substantially (Table III).

In the case of the Portugieser variety, extended skin contact time in Table III led to much higher variation of the Rac/cum as it was described for 66 commercial wines with a mean value of 1.29 and the lower and upper 95% confidence range of 0.71 and 1.87, respectively (12). In a second data set of 40

Table III. Ratio of acetylated to coumaroylated anthocyanins (Rac/cum) and sum of acylated anthocyanins (Σ acyl. anth.) in 2001 Dornfelder and Portugieser red wines after bottling.

	<i>Parameter</i>	<i>6 days</i>	<i>10 days</i>	<i>14 days</i>	<i>18 days</i>
Dornfelder	Σ acyl. anth.	13.7%	12.8%	12.5%	12.1%
	Rac/cum	3.03	3.49	3.73	3.84
Portugieser	Σ acyl. anth.	18.7%	16.8%	16.7%	16.6%
	Rac/cum	1.62	2.71	3.06	2.75

commercial wines, a similar variation was reported with a mean of 1.20 and lower and upper 95% confidence limits of 0.71 and 1.69, respectively (1, 16).

In the Dornfelder wines a similar increase of the Rac/cum could be observed, and a maceration beyond 6 days yielded Rac/cum values which were outside the upper 95% confidence range of 3.29 (12). Thus the producer may have been accused for blending a variety with a higher Rac/cum such as Cabernet Sauvignon (5) above the legal 15% limit.

Thermovinification

A substantial percentage of German red wines are vinified applying thermovinification, where crushed red grapes are heated for a short time of 2 minutes. Thermal disintegration of the skin cells facilitates a fast release of red pigments into the juice. The cooled mash is pressed and the red juice is fermented without skin and seed contact similar to white wine. The impact of thermovinification on the composition of anthocyanins, polyphenols and volatile compounds has been described for several varieties by Fischer et al. (8). The main technological variation of this semi-continuous process is based on the choice of maximal temperature between 60 and 87°C and variation of holding time between 0 and 12 hours at 40 to 45°C after the first cooling step. The higher temperature level leads to a better disintegration of the skins, enhancing color extraction, while the extended holding time increases tannin extraction.

Displaying the concentration of acylated and non-acylated malvidin-3-glucoside in a Portugieser mash in Figure 5, malvidin-3-glucoside and acetylated malvidin-3-glucoside did not change much over 12 hours of holding time at 40°C, following a 87°C heat treatment. Concurrently, the concentration of the coumaroylated malvidin-3-glucoside decreased from 82 to 52 mg/L (expressed as malvidin-3-glucoside). As a consequence, Rac/cum increased from 0.75 up to 1.23 due to the extended holding time after thermovinification. The percentage of acylated anthocyanins however remained constant (Figure 6).

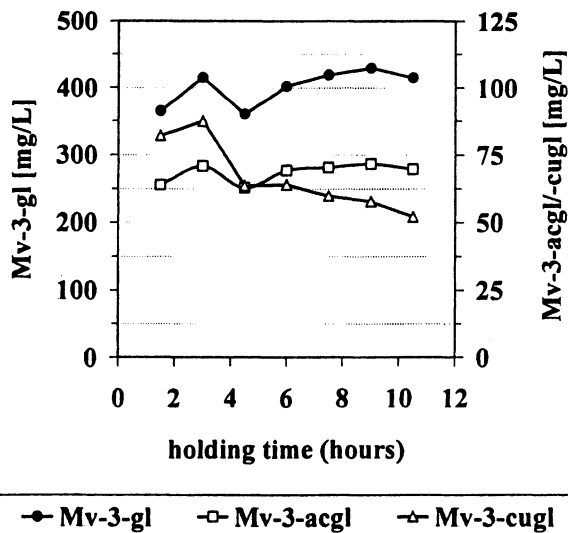


Figure 5. Change of malvidin-3-glucoside derivatives in 2002 Portugieser juice during the holding time at 40°C after a 87°C heat treatment of the grapes

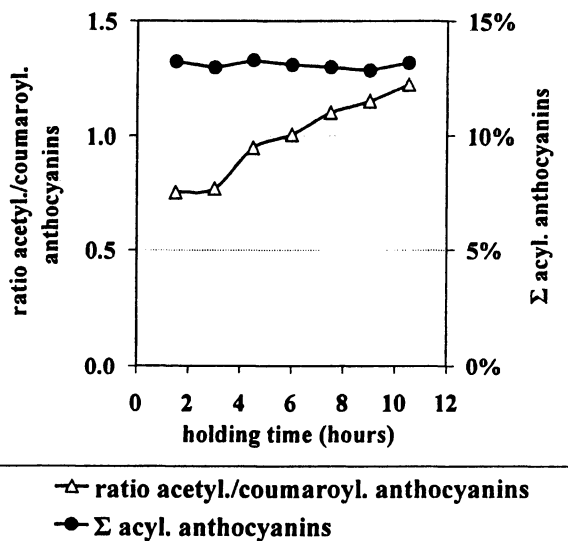


Figure 6. Ratio of acetylated to coumaroylated anthocyanins and sum of acetylated anthocyanins in 2002 Portugieser juice during the holding time at 40°C after a 87°C heat treatment of the grapes

After fermentation, maturation, and bottling of the wines, the holding time had a significant impact on the Rac/cum (Table IV), while the sum of acylated anthocyanins was rather stable. Three of the six experimental wines even slightly exceeded the reported 95% confidence range for Rac/cum in Portugieser wines (1, 12). Although the impact of the holding time is less pronounced than the impact due to extended maceration on the skins (Table III), it is very interesting to note that even short holding times of 12 hours prior to fermentation are able to modify the Rac/cum in the finished wines. Since indigenous enzymes had been inactivated by the 87°C treatment in the grapes, enzymatic oxidation can be excluded as a possible explanation for the decrease of coumaroylated malvidin-3-glucoside. Thus the elevated temperature of 40°C may have enhanced chemical oxidation of the coumaric acid moiety, diminishing the coumaroylated malvidin-3-glucoside compared to acetylated or non-acylated malvidin-3-glucoside. Absence of ethanol during the holding time may have also hindered the extraction of the coumaroylated malvidin-3-glucoside versus the acetylated or non-acylated malvidin-3-glucoside.

Enzyme treatment

According to the data shown in Table IV, addition of a pectinase which was free of any relevant side activities slightly decreased the sum of acylated anthocyanins and increased the Rac/cum after the short holding times of 0 and 4 hours, but not any further during the extended holding period of 12 hours.

Table IV. Ratio of acetylated to coumaroylated anthocyanins (Rac/cum) and sum of acylated anthocyanins (Σ acyl. anth.) in thermovinified 2002 Portugieser red wines after bottling

<i>Portugieser</i>	<i>Parameter</i>	<i>0 h</i>	<i>4 h</i>	<i>12 h</i>
without enzyme	Σ acyl. anth.	24.6%	23.3%	20.3%
	Rac/cum	1.10	1.21	1.87
with pectinase	Σ acyl. anth.	19.9%	20.0%	19.0%
	Rac/cum	1.83	1.77	2.02

In order to summarize the impact of red wine technology on the anthocyanin composition, a principal component analysis was conducted using the concentration of 9 anthocyanin derivatives and shikimic acid as well as the percentage of acylated anthocyanins (Σ acyl. anth.) and the ratio of acetylated/coumaroylated anthocyanins (Rac/cum), which was determined in 7

experimental wines from 2001 and 13 from 2002, all made from the variety Portugieser (Figure 7).

In general, wines from the 2001 vintage are located in the left half of the graph indicating lower concentration in all anthocyanins compared to vintage 2002. At 87°C and after extended holding times during thermovinification the scores of the wines (TV) move towards the right side of the PCA due to elevated concentration of all anthocyanins. Decreasing holding time and temperature to 60°C shift the wines to the left side, indicating lower extraction of anthocyanins. Most of the thermovinified wines (TV) are plotted in the upper half of the PCA plane while the majority of the wines made by fermentation on the skins (SM) are located in the lower half of the graph. Extending the skin contact time during fermentation on the skins (SM) moves the wines downward, which correlates with a diminished sum of acylated anthocyanins and enhanced Rac/cum.

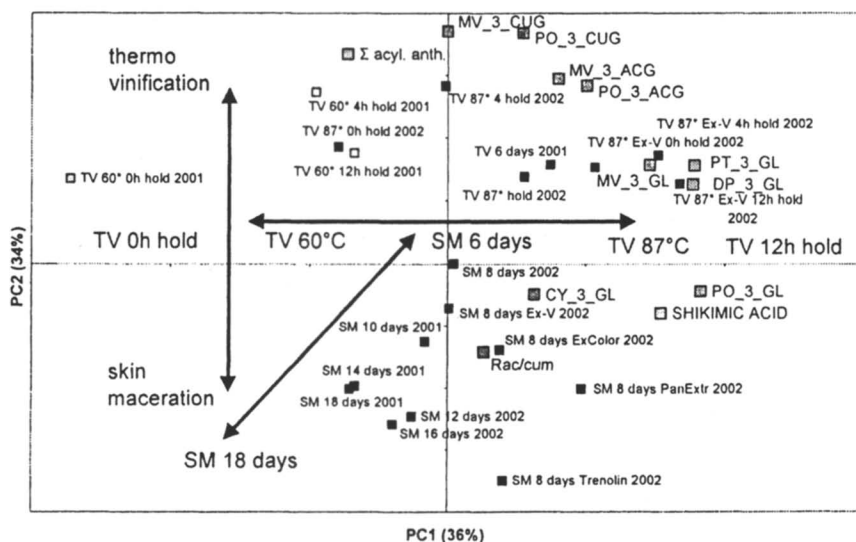


Figure 7. Principal Component Analysis (PCA) of 20 experimental wines of the Portugieser variety. Larger squares and capital letters denote analytical variables (loading); the smaller squares indicate the scores of the individual experimental wines (TV=Thermovinification varying in holding time of 0, 4, 12 hours and temperature of 60 and 87°C; SM skin maceration varying in skin contact of 6, 10, 12, 14, 18 days and the addition of enzymes).

Examining the loadings of the phenolic parameters, both coumaroylated and acetylated anthocyanins, respectively, plot closely together. They correlate positively with the sum of acylated anthocyanins (Σ acyl. anth.), but negatively with the ratio of acetylated to coumaroylated anthocyanins (Rac/cum). All five non-acylated anthocyanins are highly correlated among each other, especially malvidin-, petunindin-, and delphinidin-3-glucoside.

Addition of 4 commercial pectinases during the 8 day skin maceration trials (Lallzyme Ex-V and Panzym Extract, Lallemand, Montreal, Canada; Trenolin Rouge DF Erbslöh, Geisenheim Germany; Rapidase ExColor, DSM, Netherlands) moves the scores of the wine in the negative direction of PC2, comparable to the impact of extended skin contact of 12 or 16 days. Thus pectolytic enzymes seem to be a useful tool to reduce skin contact time without losing extraction of anthocyanins. The strongest impact was obtained for the Trenolin Rouge DF enzyme, shifting the wines in the opposite direction than the acylated anthocyanins. Due to the strong acetyltransferase activity in this enzyme preparation both acetylated anthocyanins were strongly diminished and the Rac/cum was close to zero. This result coincides with the report by Eder et al. (7) where Rac/cum in Zweigelt was diminished from 1.04 to 0.36 and in Trollinger from 0.24 to 0.08 by the use of Trenolin Rouge DF. While the Lallzyme Ex-V enzyme had no impact on Rac/cum, the Panzym Extract and the Rapidase Ex Color enzymes increased the ratio by 0.55 and 0.7, similar to the effect of extended skin maceration of 12 and 16 days. Using Rapidase Ex Color in Trollinger a comparable shift in Rac/cum from 0.24 to 0.32 was observed (7).

Some publications state that anthocyanin composition is only altered to a minor degree by viticultural and enological practices and therefore their use for authentication purpose is not limited (7, 16). However, only a few authors actually investigated the impact of grape processing and wine making on the anthocyanin composition with respect to the authentication criteria sum of acylated anthocyanins and Rac/cum. For infection by *Botrytis cinerea* Eder et al. (7) reported a substantial decrease of acylated anthocyanins from 9.2% to 5% for the Trollinger cultivar and 20.6% to 17.3% for the Austrian crossing Zweigelt. At the same time Rac/cum was enhanced for Trollinger from 0.24 to 0.74, while no change was observed for Zweigelt. The same authors studied the impact of 9 yeast strains in Zweigelt observing a variation in sum of acylated anthocyanins from 17.0% to 22.6% and for Rac/cum between 2.03 and 2.77. These results are supported by Morata et al. (15) who reported that adsorption of anthocyanins to the yeast cell wall varies among different yeast strains and specific anthocyanin structure. The yeast membrane is mainly composed of oligomeric polysaccharide and mannoproteins. Due to differences in polarity, anthocyanins are not equally bound to the yeast membrane and peonidin-3-glucoside had the strongest affinity of the five non-acylated anthocyanins (18).

Conclusion

Although anthocyanin composition of grapes is genetically determined, grape processing and wine making has a significant impact on the authentication criteria based on anthocyanins species. Despite the fact that only authentic varietal grapes were used for experimental wine making and applying only legal and state of the art enological treatments, principal component analysis of the experimental wines revealed a substantial dispersion. While the acetylated malvidin-3-glucoside behaved similar to the non-acetylated malvidin-3-glucoside, the coumaroylated derivative decreased much faster and changed the acetylated to coumaroylated ratio over time ratio. Thus some of the experimental wines were outside the postulated confidence intervals of the acetylated to coumaroylated ratio, confounding the verification of varietal authenticity.

In conclusion, the modifying impact of red wine technology such as enzyme treatment, applied temperature during thermovinification and duration of skin contact should gain much more consideration in the verification of varietal authenticity in order to protect both, wine producers and wine trade, against false alarms.

Acknowledgement

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Chapter 17

Assignment of the Regional Origin of Cherry Brandies by Stable Isotope Analysis

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The applicability of stable isotope analysis to assign the geographic origin of distilled beverages has been investigated using “Schwarzwälder Kirsch”, i.e., a cherry brandy from the Black Forest area in Germany, as an example. Isotope ratios were determined in authentic cherry mashes as well as in cherry brandies provided by distillers from specified locations. Analysis of distillate fractions revealed that distillations in a pot still proceed with vapor pressure isotope effects for the carbon and the hydrogen isotopologues of ethanol. It could be demonstrated that these isotope fractionations do not impair the applicability of stable isotope analysis for the authenticity assessment of cherry brandies. The first data sets from two vintages indicate that, in principle, an assignment of the origin of cherry brandies on the basis of stable isotope data is possible. However, multi-element analysis in combination with efficient statistical methods is required.

Introduction

Stable isotope analysis has developed into one of the most elegant and efficient tools for authenticity assessment of foods and food ingredients (1). Particularly in the area of wine analysis, the isotope signature of ethanol has been shown to be a useful indicator for detection of adulterations and the determination of origin (2,3). Assessments are based on two approaches: (i) the determination of the overall $\delta^{13}\text{C}$ -value of ethanol and the $\delta^{18}\text{O}$ -value of wine water by means of isotope ratio mass spectrometry (IRMS), and (ii) the analysis of the ratios $(\text{D}/\text{H})_{\text{I}}$ and $(\text{D}/\text{H})_{\text{II}}$ of ethanol by means of site-specific natural isotope fractionation-nuclear magnetic resonance (SNIF-NMR[®]) (4-6).

Stable isotope analysis has also been applied to the assessment of spirits obtained from grains or fruits. The data have been used to differentiate the botanical source of ethanol (6-13). The objective of this current study was to demonstrate the suitability of stable isotope analysis to assign the origin of fruit brandies. Cherry brandies from the Black Forest were selected as example. The Black Forest area situated in the Southwestern part of Germany is characterized by a mild climate and is one of the major cherry-growing regions. The cherry brandies from this location are of premium quality and their reputation is reflected in higher prices. The aim of the project was to differentiate these products from cherry brandies originating from Northern Italy (Alto Adige), an area just south of the Alps possessing different soil and climate conditions. Average maximum daily temperatures in this region are approximately 3 °C higher than those in the Black Forest area (14).

One of the key differences between the production of wine and spirits is the distillation step. Distillations in the course of commercial fruit brandy production are performed using batch stills. They consist of a distillation pot in which the mash is gently heated in a kind of water bath, a distillation column and a water-cooled condenser (15). The distillation column may be equipped with up to three column plates and a so-called dephlegmator, a device placed at the upper part of the column to increase the ethanol content in the vapor phase by partial condensation of water (15).

It has been described in numerous studies that processes involving vaporization and condensation are accompanied by isotopic fractionations resulting in so-called vapor pressure isotope effects (VPIE) (16). A depletion of the heavier isotopologue in the vapor phase compared to the liquid is called "normal VPIE". This type of VPIE is always observed in kinetic (one-way and irreversible) processes (17,18). Under thermodynamic equilibrium conditions reversible phase transitions may also result in the enrichment of the heavier isotopologue in the vapor phase (19). Such "inverse VPIE" have for example been observed for the carbon and hydrogen isotopologues (at the methyl and

methylene sites) of ethanol in the course of distillations using spinning band columns (18,20).

Owing to these effects, the first part of this study was devoted to investigations of variations in the overall carbon isotope ratio and the deuterium isotope ratios of ethanol from cherry mash as a function of the distillation yield using a traditional pot still. The elaborated data should form the basis to rule out a potential impairment of the applicability of isotope analysis for the quality assessment of fruit brandies. In the second part, data on isotope ratios in cherry mashes and in the corresponding brandies have been determined. First approaches to differentiate cherry brandies from different regions on the basis of isotope data are presented.

Experimental Conditions

Material

Unfermented cherry mash and the corresponding cherry brandies were provided by distillers from the Black Forest area (vintage 2003: n=22; 2004: n=12), from Franconia (vintage 2003: n=5; 2004: n=5) and from Northern Italy (Alto Adige) (vintage 2003: n=3; 2004: n=6). The distillates were obtained under conditions as they are commonly applied in fruit brandy production.

Distillation

Fermented cherry mash (130 L) was distilled in a 150 L-pot still (Holstein Export D) manufactured by Arnold Holstein GmbH, Markdorf (Germany). The distillation apparatus (pot diameter: 800 mm) was equipped with a column (diameter: 400 mm) containing three column plates and a dephlegmator (filled 1/3). The distillate was separated into 38 fractions (each 500 mL). Each of these fractions was analyzed by IRMS and SNIF-NMR[®]. In addition, aliquots of fractions 4-18 (middle cut 1) and fractions 4-22 (middle cut 2) were recombined and also subjected to stable isotope analysis. Triplicate distillations were carried out under strictly identical conditions.

Elemental Analysis Isotope Ratio Mass Spectrometry

$^{13}\text{C}/^{12}\text{C}$ of sugar, $^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$ and $^{34}\text{S}/^{32}\text{S}$ of the pulp were measured by Elementar elemental analyzer Vario EL III (Elementar Analysensysteme GmbH, Hanau, Germany) coupled to an isotope ratio mass spectrometer (IRMS) AP

2003 (GVI Instruments Ltd. Manchester, UK). D/H ratios of the pulp were measured using a Delta XL plus IRMS coupled with a high temperature pyrolysis unit (Thermo Instruments GmbH, Dortmund, Germany). The following standards with known ratios were used: standard casein (Sigma-Aldrich, analytical grade) which had been calibrated in a European research project (SMT4-CT2236-1998) for $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$, and later for D/H and $^{34}\text{S}/^{32}\text{S}$ versus official reference materials (PE F-1 and NIST-22, V-CDT and silver sulfide, respectively). The values are reported in the δ -scale (‰) according to the corresponding international standards (PDB, NBS-22, V-CDT, air N_2). To check the reliability of the analyses, additional in-house reference materials (wheat flour, lactose, sucrose) of known isotopic compositions were used.

Gas Chromatography combustion Isotope Ratio Mass Spectrometry (GC-c-IRMS)

A Finnigan delta S isotope ratio mass spectrometer coupled by an "open-split" via a combustion interface to a Varian gas chromatograph (GC) was used. The GC was equipped with a Poraplot U fused silica capillary column (25 m \times 0.32 mm i.d.; d_f 0.10 μm). The following conditions were employed: 0.5 μL splitless injection (250 $^\circ\text{C}$); temperature program, raised from 60 to 190 $^\circ\text{C}$ at 10 $^\circ\text{C}/\text{min}$; helium flow, 3 mL/min; combustion interface temperature, 940 $^\circ\text{C}$; reduction furnace 600 $^\circ\text{C}$. The effluent from the GC passes the combustion furnace through a ceramic tube (Al_2O_3 , $l = 320$ mm; 0.5 mm i.d.). The separated compounds eluting from the GC column are converted into CO_2 in the combustion interface. Analysis is performed in the mass spectrometer by simultaneous recording of masses 44 ($^{12}\text{C}^{16}\text{O}_2$), 45 ($^{13}\text{C}^{16}\text{O}_2$) and 46 ($^{12}\text{C}^{16}\text{O}^{18}\text{O}$). System stability check was carried out routinely by measuring a JRC (Joint Research Centre of the EU, Ispra, Italy) standard ethanol sample. The isotope ratio $^{13}\text{C}/^{12}\text{C}$ is expressed in per mil (‰) deviation relative to the Vienna Pee Dee Belemnite (V-PDB) international standard. Results are expressed in $\delta^{13}\text{C}$ -values as

$$\delta^{13}\text{C} [\text{‰}] = \left(\frac{R_{\text{sample}} - R_{\text{V-PDB}}}{R_{\text{V-PDB}}} \right) \cdot 1000$$

where R is the isotope ratio $^{13}\text{C}/^{12}\text{C}$. Triplicate analyses were carried out and standard deviations were calculated.

The $^{18}\text{O}/^{16}\text{O}$ -isotope ratio of water was determined by IRMS using the ions m/z 46 ($^{12}\text{C}^{16}\text{O}^{18}\text{O}$) and m/z 44 ($^{12}\text{C}^{16}\text{O}_2$) which are obtained after equilibrium of the isotope exchange of water and carbon dioxide. The exchange reaction $^{12}\text{C}^{16}\text{O}_2 + \text{H}_2^{18}\text{O} \leftrightarrow ^{12}\text{C}^{16}\text{O}^{18}\text{O} + \text{H}_2^{16}\text{O}$ proceeds via the solved hydrogen carbonate and is temperature dependent. The carbon dioxide in the vapor phase

is used for analysis. For equilibration, the sample flasks are filled with 2 to 5 ml sample, evacuated and carbon dioxide is introduced. The sample bottles are placed in a thermostatically controlled water bath at 25 °C. After equilibration (4-12 hrs.) the carbon dioxide of the sample bottles is transferred to the mass spectrometer through a cryogenic trap maintained at -80 °C to remove water vapor and ethanol. The measurements are performed versus calibrated laboratory standard water. The relative difference of the ion intensities of m/z 46 and 44 (I46/I44) between the samples and standards are measured in ‰ and expressed in the relative difference $\delta^{18}\text{O}$ versus the Vienna Standard Mean Ocean Water (SMOW). The method is in accordance with EC-Regulation 822/97 (21).

Thermo Ionization Mass Spectrometry (TIMS)

For $^{87}\text{Sr}/^{86}\text{Sr}$ -isotope analysis about 100 to 500 mg of the sample (depending on the strontium content) is weighed into quartz-crucibles. While liquid samples like juice or the soluble fraction firstly must be concentrated and dried for approximately 10 hours under ruby light, pulp samples can directly be ashed by heating slowly up to 850 °C and then holding at that temperature for about 5 hours. The residue is dissolved in HNO_3 (conc.) and evaporated to dryness. 3 mL of 3 N HNO_3 are added. Possible insoluble components are removed by centrifugation. For the chemical separation small columns with 50 μL resin-volume are used. The resin used is an extraction chromatographic material called "Sr-Spec" which can be obtained from Eichrom[®] (ordering number SR-B25-S). After loading the sample onto the columns, the columns are washed two times with 0.1 mL and three times with 0.3 mL of 3 N HNO_3 to elute interfering elements like Ba and, especially Rb. Sr is eluted with 0.4 mL of 0.05 N HNO_3 . The Sr fraction is collected in small Teflon beakers and evaporated to dryness. The complete chemical procedure is carried out in a clean-laboratory and only ultra pure reagents are used.

For the measurement of Sr isotope-ratios tungsten single filaments are used. First 1 μL of Ta-Fluoride ("Birk'sche Lösung") is loaded onto the filaments to improve ionization of Sr during the measurement. After that the sample is dissolved in 2 μL of 2 N HCl and is also loaded onto the filaments. The measurements are performed with a MAT 261 TIMS (Finnigan). To control the reproducibility, a certified isotopic standard (SRM 987) is measured with every run.

SNIF-NMR[®] (Site-specific Natural Isotope Fractionation Deuterium Nuclear Magnetic Resonance)

A Deuterium Nuclear Magnetic Resonance (ARX 400, Bruker), fitted with a specific deuterium probe tuned to the characteristic frequency V_0 of the field B_0

having a proton decoupling channel (B2) and field-frequency stabilization channel (lock) at the fluorine frequency was used. For each sample, $(D/H)_I$, $(D/H)_{II}$ and R were measured according to EC Regulation No 2676/90 (22). The results are expressed in ppm.

Limits of repeatability

10-fold analysis of reference samples was performed according to the procedure described in Commission Regulations (EEC) No 2676/90 (22) and (EC) No 440/2003 (23). For the determination of $\delta^{13}\text{C}$ a standard ethanol sample from the JRC and for (D/H)-measurements an ethanol-water mixture were used. For each isotope ratio the standard deviation (s) and the corresponding limit of repeatability (r) were calculated in accordance with ISO 5725 (24) using the following equation: $r = 1.96 \cdot \sqrt{2} \cdot s$.

Results and Discussion

Influence of the distillation on isotope ratios of ethanol

Fermented cherry mash was subjected to distillation in a type of pot still commonly applied in fruit brandy production. The distillate was separated into 500 mL-fractions and each fraction was analyzed by means of IRMS and SNIF-NMR[®]. As shown in Figure 1, the distillation in the pot still proceeded with an inverse VPIE for the carbon isotopologues of ethanol. Starting from a $\delta^{13}\text{C}$ -value of ethanol in the cherry mash of -27.61 ‰, the highest $\delta^{13}\text{C}$ -value (-26.80 ‰) was observed in the first distillate fraction. Progressing distillation resulted in a significant decrease of $\delta^{13}\text{C}$ -values of the subsequently collected fractions. This inverse VPIE is in accordance with data reported for the distillation of ethanol under thermodynamic equilibrium conditions using a spinning band column (18).

To determine whether the observed isotopic fractionation might influence the applicability of stable isotope analysis to the assessment of fruit brandies, the collected 500 mL-fractions were recombined to cuts, as they are common practice in commercial fruit brandy production: foreshot, middle cut (heart) and tail cut (15). The volume of the middle cut is determined by the ethanol content of the distillate. In commercial practice, cutoff points range between ethanol contents of 65 and 50 %-vol. On the basis of these two limits, two middle cuts were prepared by re-combination of the corresponding 500 mL-fractions (Figure 1). The $\delta^{13}\text{C}$ -values determined by IRMS analysis of middle cuts 1 and 2 are shown in Table I.

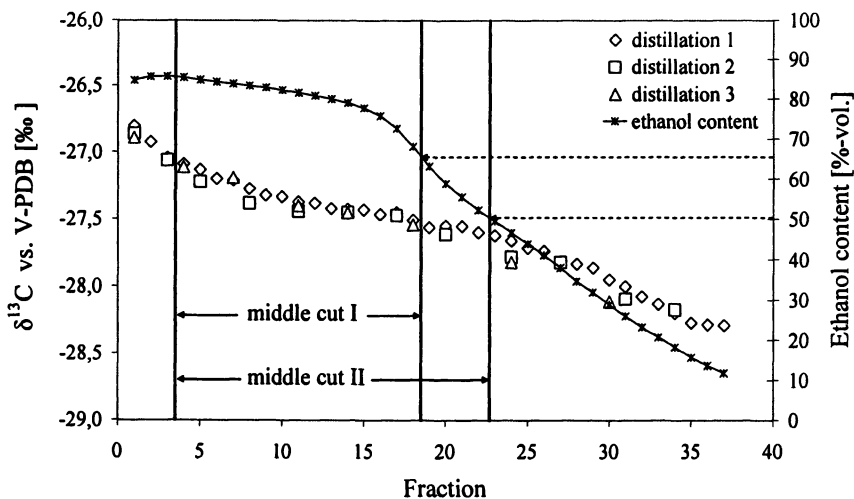


Figure 1. $\delta^{13}\text{C}$ -values of ethanol in cherry brandy as function of the distillation progress.

Table I. $\delta^{13}\text{C}$ -values of ethanol in middle cuts 1 and 2

Distillation	$\delta^{13}\text{C}$ vs. V-PDB [‰] ^{a,b}		Δ [‰]
	cut 1 (65 %-vol.)	cut 2 (50 %-vol.)	
1	-27.28	-27.34	0.06
2	-27.35	-27.41	0.06
3	-27.24	-27.32	0.08

^a Average values from three distillation experiments

^b Calculations were performed according to the following equation

$$\Pi_m = \frac{\sum_{i=a}^b (\Pi \cdot A)_i}{\sum_{i=a}^b A_i}$$

Π : $\delta^{13}\text{C}$; A: alcoholic content (%-vol.) of the particular fraction i ; a and b: first and last fraction of the distillation series. For the distillation series 2 and 3 not all fractions had been analyzed by means of isotope ratio analysis (see Figure 1); the missing data points were calculated by regression analysis (r^2 : 0.95- 0.99).

The differences ($\Delta \delta^{13}\text{C}$) between the isotope values of the two middle cuts observed in three distillation experiments range from 0.06 to 0.08 ‰. For the officially accepted method to determine $^{13}\text{C}/^{12}\text{C}$ -ratios in wine ethanol and ethanol obtained by fermentation of products of the vine a limit of repeatability (r) of 0.24 ‰ has been set (23). This limit is defined as the value below which the absolute difference between two single test results obtained on identical test material under the same conditions may be expected to lie, with a specified probability of 95 % (24). Under the experimental conditions of this study, a limit of repeatability (r) of 0.17 ‰ was determined. When comparing these limits with the differences ($\Delta \delta^{13}\text{C}$) observed between middle cuts 1 and 2, it becomes obvious that the isotope fractionations observed do not impair the applicability of the stable isotope method for quality assessment of fruit brandies if the cuts are placed in accordance with common distillers' practice.

The $(\text{D}/\text{H})_{\text{I}}$ - and $(\text{D}/\text{H})_{\text{II}}$ -ratios determined in the 500 mL-distillate fractions by SNIF-NMR[®] are shown in Figure 2A and B.

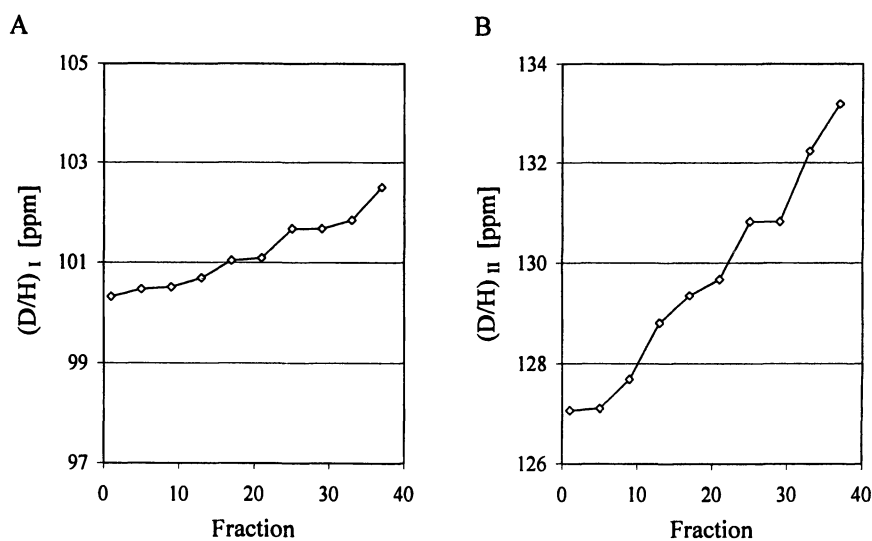


Figure 2. (D/H) -ratios of ethanol in the distillate from cherry mash in the course of distillation in a pot still.

In contrast to the behavior observed for the carbon isotopes, the first fraction exhibited the lowest (D/H) -ratios ($(\text{D}/\text{H})_{\text{I}}$: 100.43 ppm; $(\text{D}/\text{H})_{\text{II}}$: 126.96 ppm). Considering the $(\text{D}/\text{H})_{\text{I}}$ - and $(\text{D}/\text{H})_{\text{II}}$ -ratios of the starting material (100.98 ppm and 129.90 ppm, respectively), this demonstrates that the distillation in the pot still proceeds with a normal VPIC for the hydrogen

isotopologues of ethanol. This is in contrast to the data reported for distillation of ethanol using spinning band columns (18,20). The isotope ratios of the distillate increase with progressing distillation, whereby the slope of the $(D/H)_{II}$ increase is significantly higher than that of $(D/H)_I$. The data shown in Figure 2 could be confirmed in two further distillations.

In analogy to the procedure described for the carbon isotopes, the $(D/H)_I$ - and the $(D/H)_{II}$ -ratios were determined for the middle cuts 1 and 2 (Table II).

Table II. (D/H)-ratios of middle cuts 1 and 2

Distillation	$(D/H)_I$ [ppm] ^a		Δ [%]
	cut 1 (65 %-vol.)	cut 2 (50 %-vol.)	
1	100.61	100.73	0.12
2	100.00	100.16	0.16
3	100.93	101.03	0.10
Distillation	$(D/H)_{II}$ [ppm] ^a		Δ [%]
	cut 1 (65 %-vol.)	cut 2 (50 %-vol.)	
1	128.18	128.52	0.34
2	127.74	128.12	0.38
3	129.03	129.30	0.27

^a Calculations were performed according to the equation in footnote b of Table I, inserting (D/H) -ratios for Π .

As shown in Table II, the differences for the two fractions range from 0.10 to 0.16 ppm ($(D/H)_I$) and from 0.27 to 0.38 ppm ($(D/H)_{II}$). These data were compared to the limits of repeatability (r) of 0.83 for $(D/H)_I$ and $(D/H)_{II}$, calculated from the officially set standard deviation for repeatability of 0.3 ppm (22) and to the limits of repeatability determined under the conditions of this study (0.55 ppm for $(D/H)_I$) and of 0.64 ppm for $(D/H)_{II}$). Differences in isotope ratios arising from the different cuts are significantly lower than these limits.

In conclusion, the distillation of cherry mash in a type of pot still used for commercial production of fruit brandies proceeds with a fractionation of the ethanol isotopologues. The inverse VPIC observed for the carbon isotopologues is in accordance with the data reported for distillation of ethanol in spinning band columns (18). In contrast, the inverse VPIC for the hydrogen isotopologues of ethanol observed in spinning band columns (18,20) could not be confirmed. The isotope effects are the result of the normal isotope effect from hindered translations and rotations and the inverse isotope effect arising from the internal degrees of freedom, which interplay under thermodynamic equilibrium

conditions (19), and the normal VPIE characteristic for strictly kinetic conditions (17). The processes in the course of the distillation in the pot still employed in this study are characterized by a complex combination of such phenomena. At present, it is difficult to interpret the observed isotope fractionations on the basis of theoretical considerations.

Taking into account the common practice of using defined cuts of the distillate for fruit brandy production and the limits of repeatability of the methods applied, it could be demonstrated that the isotope fractionations observed do not influence the applicability of stable isotope analysis of the carbon and hydrogen isotopes of ethanol for authenticity assessment of cherry brandies.

Isotope ratios

Cherry mashes and the corresponding cherry brandies distilled under defined conditions were provided by a network of distillers from the Black Forest region and from Northern Italy. In addition, material from Franconia, a well-known wine growing region in Germany was analyzed.

Isotope ratios of the cherry mashes

Multi-element stable isotope analysis is known to be a reliable tool for the origin assignment of food. $\delta^2\text{H}$ -values of certain food components (e.g. pulp, ethanol) and $^{18}\text{O}/^{16}\text{O}$ -ratios of water primarily reflect the climatic conditions of the growing region whereas $\delta^{13}\text{C}$ -values are characteristic for botanical origin. In contrast $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ -values are typical for the particular soil and also dependent on agricultural practice (e.g. use of fertilizers) or soil erosion (1,25). In addition to the described values, the isotope ratio of strontium ($^{87}\text{Sr}/^{86}\text{Sr}$), a trace component accompanying calcium in nature, has been used to differentiate foods according to their geographical origin as it is only determined by the geological conditions and thus not affected by any climatic or anthropogenic influence (26). Different combinations of these isotope ratios have been used to assign the geographical origin of various food, such as wine, orange juice, milk, cheese, butter, or beef (1,25-30).

For the origin assignment of unfermented cherry mashes, $\delta^{13}\text{C}$ -values of the pulp and sugar, the $^{18}\text{O}/^{16}\text{O}$ -ratio of water and $\delta^2\text{H}$ -, $\delta^{15}\text{N}$ -, $\delta^{34}\text{S}$ -, and $\delta^{87}\text{Sr}$ -values of the pulp were determined. The minimum/maximum values and the medians of the isotope ratios determined in the material from 2003 are shown in Figure 3.

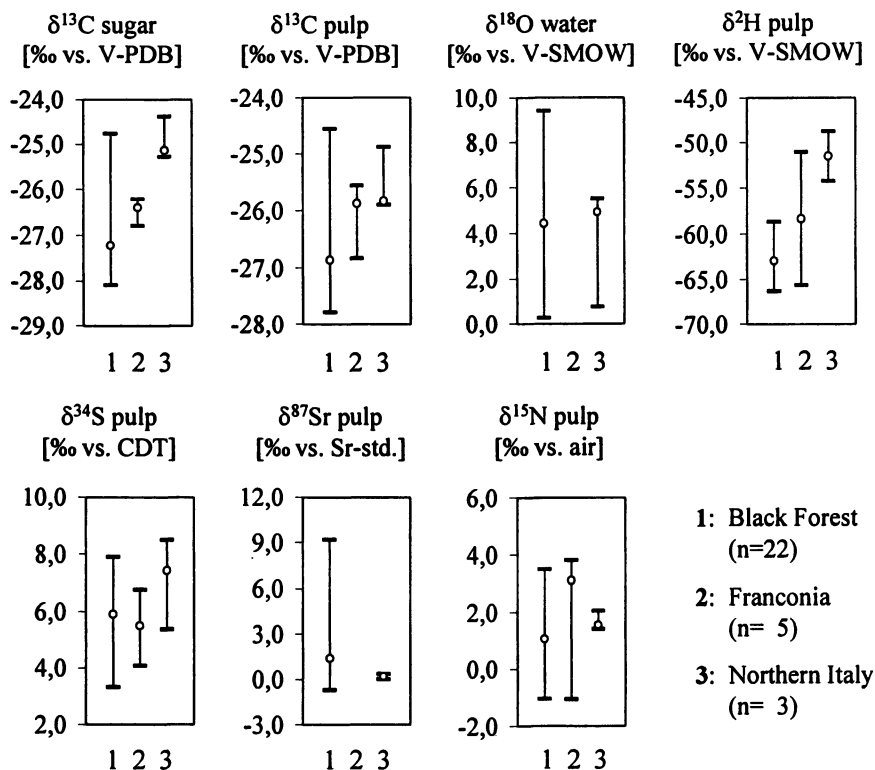


Figure 3. Isotope ratios in unfermented cherry mashes from 2003 (the upper and lower ends of the bars correspond to the maximum/minimum values determined; the open circles show the medians).

There are significant overlaps of the isotope data from the different regions. Based on single isotope ratios, no differentiation would be possible. However, Figure 4 A demonstrates that linear discriminant analysis on the basis of the $\delta^{13}\text{C}$ -values of sugar and the $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ -values of the pulp resulted in different clusters for the samples from the Black Forest and Northern Italy. The samples from Franconia showed some overlap with those from the Black Forest. When applying linear discriminant analysis to the $\delta^{13}\text{C}$ -values of sugar and the $\delta^{13}\text{C}$ -, $\delta^2\text{H}$ -, $\delta^{15}\text{N}$ - and $\delta^{34}\text{S}$ -values of the pulp of the cherries from 2004 even a separation of the fruits from Franconia from the other two growing areas was possible (Figure 4B). This improvement can be ascribed to the additional integration of the $\delta^2\text{H}$ -values, which are known to be dependent on climatic

conditions and thus the geographical origin (8). In addition, the poorer differentiation of the cherries from 2003 might also be explained by the fact that the summer of 2003 was unusually hot and dry in Central Europe, so that normally existing regional differences in climate were masked.

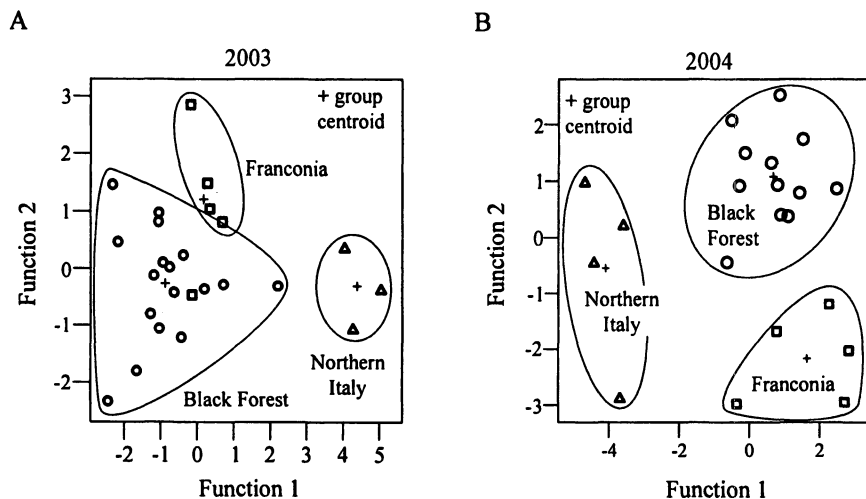


Figure 4. Linear discriminant analysis of isotope ratios of unfermented cherry mashes.

A: vintage 2003; combination of $\delta^{13}\text{C}$ sugar and $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ pulp
B: vintage 2004; combination of $\delta^{13}\text{C}$ sugar and $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, and $\delta^2\text{H}$ pulp.

Isotope ratios of the distillates

$\delta^{13}\text{C}$ -values and (D/H)-ratios of ethanol were determined in distillates from 2003 and 2004. In material from 2003 the $\delta^{18}\text{O}$ -value of water was also analyzed. Maximum/minimum data as well as the medians obtained are shown in Figure 5. $\delta^{13}\text{C}$ -values and (D/H)_I- and (D/H)_{II}-ratios were in the same order of magnitude as previously reported for cherry brandies (7,8).

For wine it has been shown that the $\delta^{13}\text{C}$ -value of ethanol is not as reliable for the assignment of the origin as the $\delta^{18}\text{O}$ -value in wine water (31,32). In general, $\delta^{13}\text{C}$ -values mainly reflect botanical origin and mode of CO_2 fixation rather than geographical origin (33). However, wines from warmer and dryer Mediterranean regions exhibit higher $\delta^{13}\text{C}$ -values than those from more humid

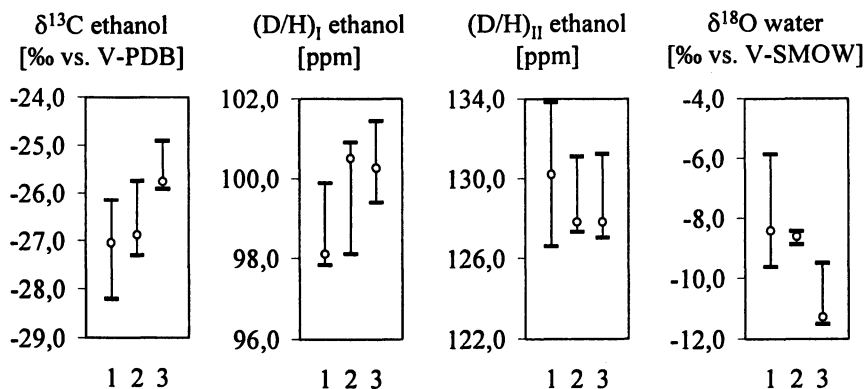
and colder regions, e.g. close to the Alps (33). A similar phenomenon has been reported for $\delta^{13}\text{C}$ -values in wines from different regions in Germany (33). For wines from Slovenia the combination of isotope analysis with chemometric methods allowed the differentiation of wines according to geographical origin. On the basis of $(\text{D}/\text{H})_{\text{II}}$ and $\delta^{13}\text{C}$ -values for ethanol a separation of wines of the coastal region from those of the continental region could be achieved. Inclusion of $\delta^{18}\text{O}$ -values into the data package subjected to principal component analysis (PCA) and linear discriminant analysis (LDA) made also possible the differentiation of wines from the continental regions Drava and Sava (34). Similar differences between stable isotope ratios of wines from continental and coastal regions were reported for wines from Croatia (35).

The lower $\delta^{13}\text{C}$ -values determined in distillates from the Black Forest area compared to distillates from Northern Italy shown in Figure 5 are in accordance with these trends. However, for wine it has been discussed that the general picture is complicated by looking at detailed results from locations: high $\delta^{13}\text{C}$ -values were found at certain places, even in regions with low average values and vice versa (33).

In the distillates from 2003 the clearest difference between isotope ratios was observed for the $\delta^{18}\text{O}$ -values of water. Mean $\delta^{18}\text{O}$ -values of water in wines from different wine-producing regions in Italy and France indicated a trend of decreasing $\delta^{18}\text{O}$ -values from the South to the North, induced by lower mean temperatures (36-38). In Germany such a regional pattern was not evident (33). Considering the above mentioned differences in average maximum daily temperatures between the Black Forest area and Northern Italy, the higher $\delta^{18}\text{O}$ -values determined in water of distillates from the Black Forest are not in agreement with the phenomena observed for wine. However, these values are also influenced by the isotope ratio of the ground water originating from precipitation and taken up by the plant. In Northern Italy the so-called altitude effect (39) caused by the surrounding alps results in a significant depletion of the ^{18}O -isotopes of the water (40), which is reflected in the lower $\delta^{18}\text{O}$ -values of the distillates.

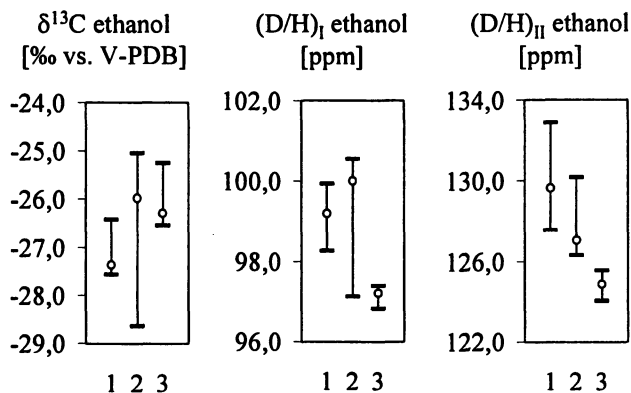
$(\text{D}/\text{H})_{\text{II}}$ -ratios give important information on environmental conditions and fermentation medium (41). In 2003 there were no differences in $(\text{D}/\text{H})_{\text{II}}$ -ratios between distillates from the Black Forest and distillates from Northern Italy. The results are consistent with isotope patterns of cherry mashes grown in a hot and dry climate, as was experienced in Central Europe in the summer of 2003. In cherry brandies of the vintage 2004 significantly higher $(\text{D}/\text{H})_{\text{II}}$ -ratios were found in the distillates from the Black Forest compared to Northern Italy. This may also be explained by the depletion in ^2H -isotopes of the ground water in Northern Italy due to the altitude effect as discussed for the $\delta^{18}\text{O}$ -values.

A



2003 1: Black Forest (n=22)
 2: Franconia (n= 5)
 3: Northern Italy (n= 3)

B



2004 1: Black Forest (n=5)
 2: Franconia (n=5)
 3: Northern Italy (n=6)

Figure 5. Isotope ratios in cherry brandies (the upper and lower ends of the bars correspond to the maximum/minimum values determined; the open circles show the medians).

Comparable to the data obtained for the cherry mashes, there are significant overlaps of the single isotope ratios for the materials from the three regions. Linear discriminant analysis of the isotope ratios determined in the cherry brandies is shown in Figure 6.

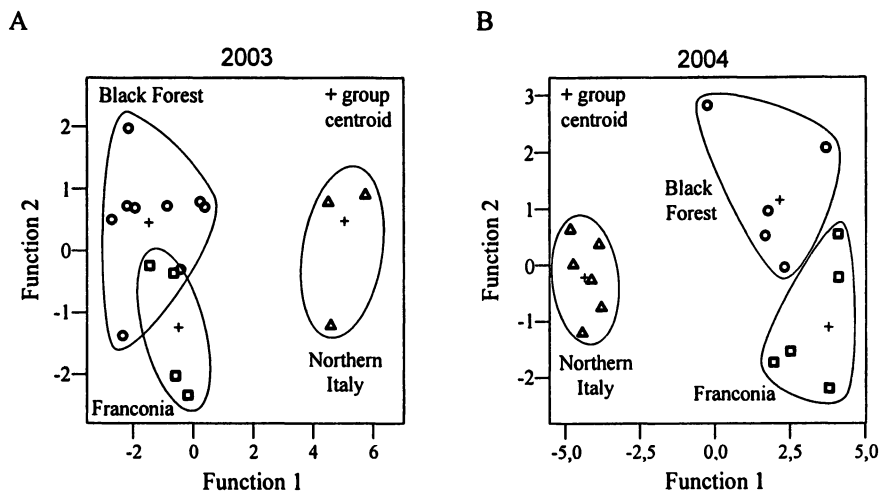


Figure 6. Linear discriminant analysis of isotope ratios of cherry brandies. *A:* vintage 2003; combination of $\delta^{13}\text{C}$, $(\text{D}/\text{H})_{\text{I}}$, $(\text{D}/\text{H})_{\text{II}}$ ethanol, and $\delta^{18}\text{O}$ water *B:* vintage 2004; combination of $\delta^{13}\text{C}$, $(\text{D}/\text{H})_{\text{I}}$, and $(\text{D}/\text{H})_{\text{II}}$ ethanol.

For the distillates from 2003 (Figure 6A) linear combination of the $\delta^{13}\text{C}$ -values and the (D/H) -ratios of ethanol and the $\delta^{18}\text{O}$ -values of water resulted in separation of the clusters obtained for the material from the Black Forest and Northern Italy. Comparable to the data obtained by LDA of the isotope ratios in the mashes, distillates from the Black Forest and from Franconia could not be separated. In 2004 (Figure 6B) the discrimination between the distillates from the Black Forest and Franconia on one hand and those from Northern Italy on the other hand was even more pronounced although the $\delta^{18}\text{O}$ -values of water could not be included because these data were not available for the complete set of samples. A preliminary assessment taking into consideration distillates from 2004 for which $\delta^{18}\text{O}$ -values were also available indicated that a differentiation of distillates from Northern Italy and those from the Black Forest/Franconia regions could be achieved even if the data from the vintages 2003 and 2004 are combined.

In conclusion, the data demonstrate that in principle an assignment of the origin of cherry brandies on the basis of stable isotope data is possible. However, multi-element analysis in combination with appropriate statistical methods is necessary. The results elaborated so far should be extended by analysis of further samples from different harvests.

Acknowledgement

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Chapter 18

Authenticity: The Case of Tequila

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Agave tequilana Weber azul variety, a CAM (crassulacean acid metabolism) plant, is the only raw material used to make Tequila, the most well known distilled spirit in Mexico with origin denomination followed in importance by Mezcal, Sotol, Bacanora, and Sisal. All of these spirits are obtained from the fermentation of agavins from different *Agave* species. In the last decade, Tequila consumption has increased tremendously worldwide and its authenticity is of a high concern to Mexican government and consumers. Authenticity studies of Mexican distilled spirits have been a priority in our group for many years. The case of Tequila is presented in this work. To establish the authenticity of Tequila, different analytical studies have been performed including SPME-GC-MS profiles, NIR-SIMCA determinations, GC-O, and GC-IRMS. Blanco, Reposado, and Añejo Tequilas types of both categories (100% and mixed) were used in all determinations. SPME-GC-MS results demonstrated the presence of volatile compounds that permitted to establish some qualitative and quantitative differences among all Tequila types. Tequila types were clearly and easily grouped by NIR-SIMCA measurements but better recognition and validation were found for the Reposado type. By GC-O analyses, the most potent volatiles of authentic Tequilas based on Charm values (numbers in the parentheses) were, an unknown (16956), phenylethyl alcohol (7771), butanol-3-methyl (6515), vainillin

(5510), and a terpenoid (4733). Finally, authenticity of all types and categories of Tequilas was possible using stable isotope determinations. The measured $\delta^{13/12}\text{C}$ (-12.05 to -14.82‰) values were within the CAM range isotopic values which undoubtedly allowed to establish the plant origin, *Agave tequilana* Weber azul variety.

Introduction

What is a true tequila? An authentic Tequila is a Mexican alcoholic beverage elaborated with and only with *Agave tequilana* Weber azul variety. *A. tequilana* may be grown in 5 different states in Mexico (Jalisco, Guanajuato, Michoacan, Nayarit, and Tamaulipas). Tequila is the most well known Mexican traditional spirit with an origin denomination. Between 1996-1998 a world-wide “Tequila Boom” was experienced, which fortunately is still alive, however in a lesser intensity. As a consequence of this boom, other traditional or ethnic distilled beverages such as Mezcal, Sotol, Bacanora, and Sisal from different states of Mexico (Oaxaca, Chihuahua, Sonora, and Yucatan, respectively and different Agave species (Figure 1) have gained popularity.

Tequila production reached its highest level in 1999 with 190.6 million liters with a 40% of alcohol, of which 61.5 million were 100% Tequila. Even if production has decreased, it is still at levels higher than before the boom. In 2000, 98.8 million liters were exported and this amount has been increasing despite the drop in production.

How Tequila is Produced

Tequila elaboration involves a few stages:

1. Harvesting of at least 6 years old plants of *Agave tequilana* Weber azul variety, grown in either of the five states mentioned above.
2. Cooking of Agave pines for 36 h depending on the type and size of a company. During this stage the fructans (Agavins) are hydrolyzed.
3. Fermentation of 100% Agave juice to make 100% Tequila or fermentation of mixed juice (51% sugars from Agave and 49% from other sources) to make mixed Tequila.
4. Double distillation. At this point the Blanco Tequila is obtained.
5. Blanco Tequila can be matured in barrels up to 6 months to produce Reposado Tequila.
6. Blanco Tequila can also be aged for > 6 months to generate Añejo Tequila.

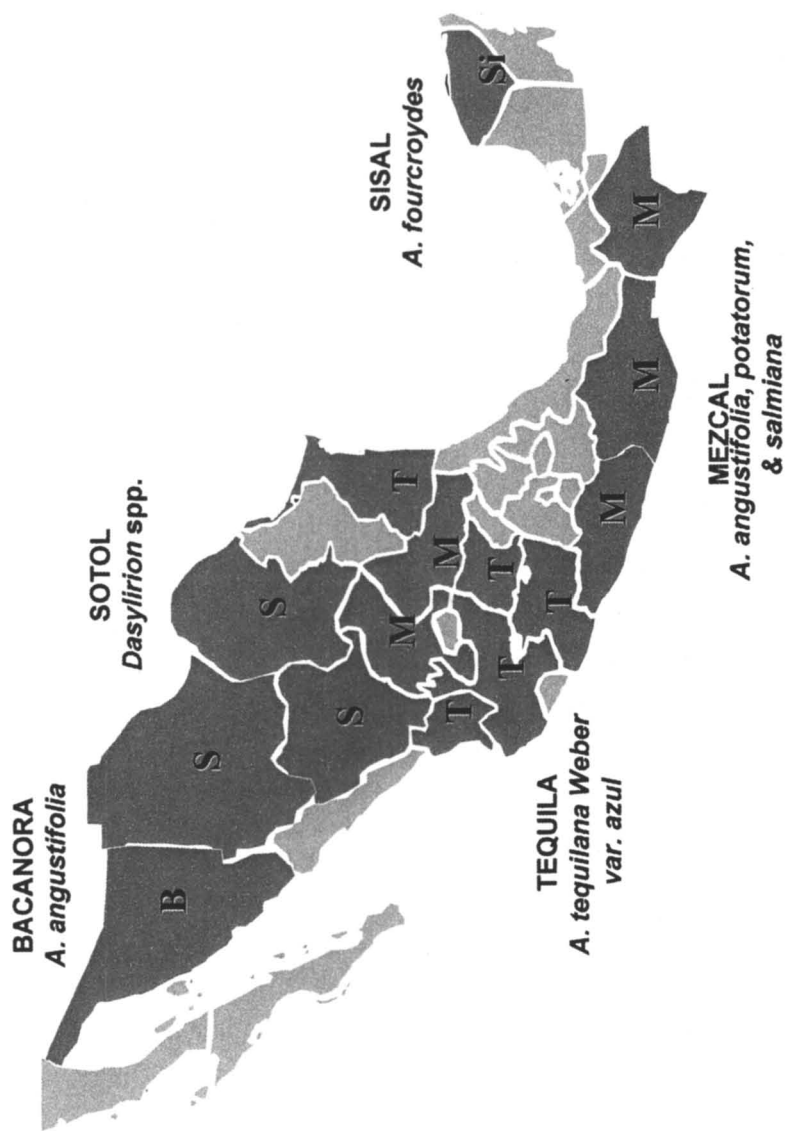


Figure 1. Distribution of Mexican Traditional Spirits. Tequila (T), Mezcal (M), Sotol (S), Bacanora (B), and Sisal (Si).

Evolution of Tequila Studies

Tequilas and other Mexican ethnic beverages have been studied for at least a decade by our group. The evolution of these investigations are presented below in a chronological order:

- 1995 – Volatile profiles of Blanco, Reposado, and Añejo Tequilas (1)
- 1997 – Agave thermal process (Maillard reaction) (2,3)
- 1998 – GC-O Aromagrams (4)
- 2000 – NIR-SIMCA Analyses (5)
- 2000 – SPME-GC-MS of Tequila, Mezcal, and Sotol (6-8)
- 2000 – Stable isotope assessment of Tequilas (9)
- 2001 – SNIF-NMR determinations (10)
- 2001 – SPME of Agave species (11)
- 2003 – Stable isotopes of Agave species (12,13)
- 2004 – FTIR-SIMCA of Tequilas from different companies (14)
- 2005 – Multivariate analysis of FTIR tequilas (15)

A summary of the results of some of these studies will be presented.

GC-O Studies

Liquid-liquid extracts of authentic Tequilas, Blanco, Reposado, and Añejo were obtained and analyzed by gas chromatography coupled to olfactometry (GC-O). Unique aromagram profiles allowed the possibility to establish differences among aged (Reposado and Añejo) and non aged Tequilas (Blanco) (4). Figure 2 shows the aromagram and chromatogram of a Blanco Tequila with Kovats index from 1400 to 1850. Figure 2 reveals the complexity of the same sample and shows the different esters and terpenes which give the Tequila a floral aroma.

SPME-GC-MS of Mexican Traditional Beverages

SPME (solid phase microextraction) coupled to GC-MS (gas chromatograph-mass spectrometry) is a highly innovative analytical tool, with high sensitivity and reproducibility that allows the determination of volatiles in many foods, even liquid foods or beverages. Therefore, the main aim of this study was to establish the potential of this technique for the authenticity of three Mexican alcoholic beverages. The potential of SPME-GC-MS technique has been demonstrated by the results obtained in this study. The results listed in Table 1 indicate that in all beverages, there are plenty of similarities as well as

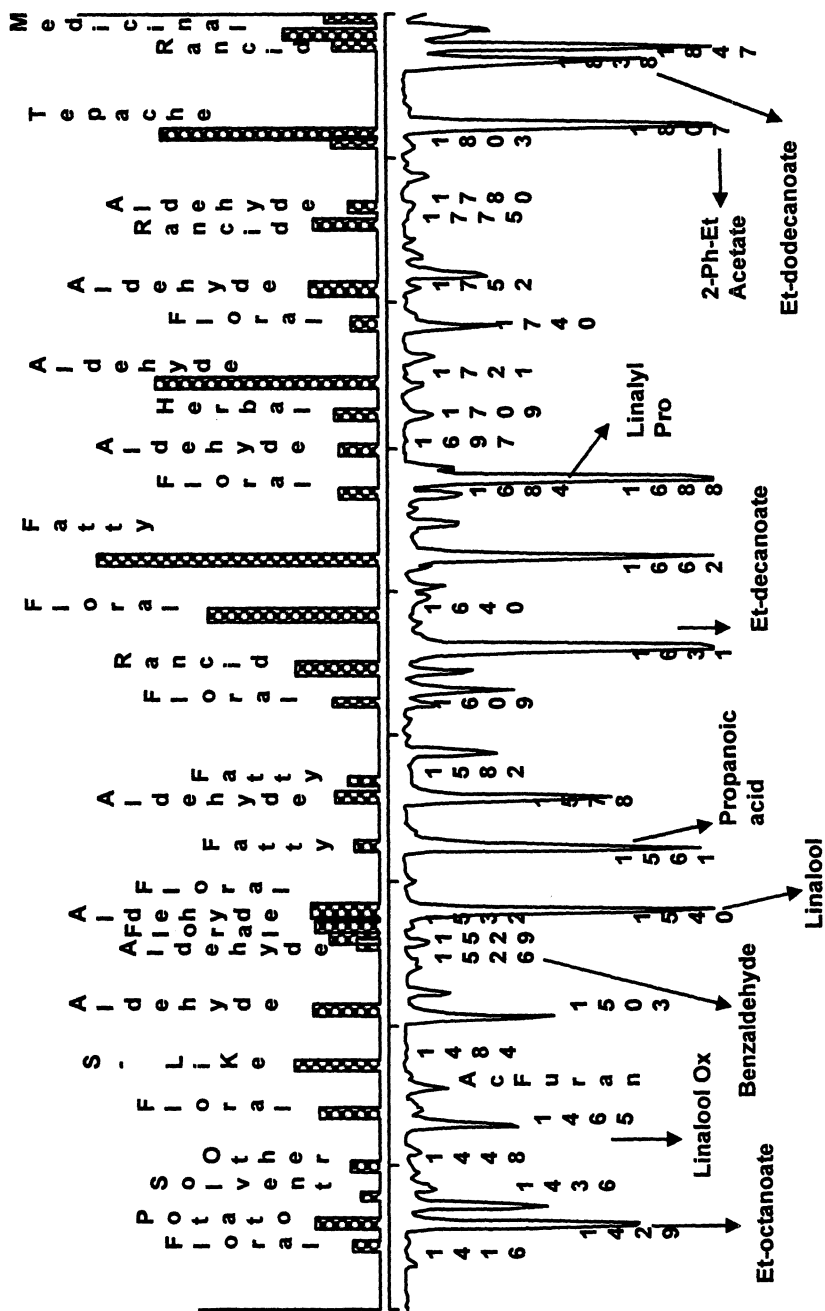


Figure 2. Section of an aromagram and chromatogram of a Blanco Tequila from 1400 to 1850.

Table I. Main compounds in Sotol (*Dasyilirion* spp.), Mezcal (*A. angustiolia*, *A. salmiana*, and *A. potatorum*), and Tequila (*A. tequilana* Weber azul variety)^a. The abundance are the averages of six different samples of each type of beverage and the compound was reported if it were present at least three times.

Rt ^b	Compound	Sotol	Mezcal	Tequila
1.00	Ethyl acetate	1376±85	943±30	395±26
1.17	Methanol	1643±101	735±68	709±70
1.99	Ethanol ^c	149±43	147±39	118±20
3.03	2-Butanol	112±33		
3.14	Butanoic acid EE ^d			45±8
3.26	Propanol	677±18	487±21	393±18
4.90	2-Me-propanol ^e	683±24	875±19	765±13
5.35	3-Me-butanolacetate	25±9	124±57	
5.49	3-Carene			26±5
7.18	Butanol	13±4	18±6	
7.78	Cyclopentanone	27±10	38±11	
8.20	3-Me-cyclopentanone	13±57	6±4	
9.00	Hexanoic acid EE	68±24	22±10	29±8
9.21	2/3-Me-butanol ^f	341±98	55±34	602±78
10.83	Pentanol	17±8		
11.09	2-Me-3(2H)-furanone	44±10	25±9	
12.22	2-Butanone-3-OH	95±47	25±11	
14.70	Propanoic acid 2-OH EE	322±59	515±87	279±45
14.95	Hexanol	42±9	6±2	
17.60	Octanoic acid EE	793±90	543±57	604±59
19.00	Acetic acid ^f	337±50	429±39	192±12
19.80	Furfural	210±51	222±23	109±21
21.45	Ethanone-1-(2-furanyl)	59±34	26±19	40±13
22.05	Nonanoic acid EE		68±28	
22.78	Propanoic acid	8±3	25±3	7±2
22.95	Linalool			714±102
23.36	Octanol		6±2	8±2
23.85	2-Me-propanoic acid	73±58	54±19	47±15
24.30	5-Me-furfuraldehyde	117±33	271±24	139±9
26.15	Decanoic acid EE	904±38	610±31	741±24
26.32	Butanoic acid	103±39		
27.74	3-Me-butanoic acid	53±12	22±10	33±12
27.81	2-Me-furan	16±8	30±9	
28.80	Terpineol	53±11	45±10	182±20

Table I.- *Continued.*

Rt ^b	Compound	Sotol	Mezcal	Tequila
30.14	Naphthalene	247±29	76±31	
31.46	Decanol		8±2	15±2
33.18	Acetic acid 2-phenyl EE	64±14	88±17	112±27
33.80	Dodecanoic acid EE	69±10	35±7	28±7
34.10	1-Me-naphthalene	70±8	2±1	
34.15	Hexanoic acid		2±1	2±1
35.10	2-Me-naphthalene	5±2		
35.92	Phenylethanol	5±3	37±7	38±5
37.05	2-Methoxy phenol		3±1	
38.11	Phenol	34±7	87±4	
38.50	4-Et-2-methoxy phenol [§]	11±6		
38.60	Tetradecanoic acid EE	106±15	43±7	
38.64	Nerolidol			76±10
39.05	Octanoic acid		42±8	116±13
42.42	Hexadecanoic acid EE	241±20	114±25	87±11
42.82	Decanoic acid		37±10	159±27

^a Relative abundance (10^{+1}). ^b Retention time for identified compounds. ^c 10^{+4} .

^d EE=Ethyl ester. ^e Me=Methyl. ^f 10^{+2} . [§] Et=Ethyl.

differences, probably due mainly to the raw ingredients as well as the elaboration. Sotol presents the largest number of components followed by Mezcal and Tequila. The presence of lesser compounds in Tequila might be due to the loss of volatiles during its elaboration since it is only of the three beverages passed through activated carbon before bottling. Figure 3 shows that esters, alcohols, and acids were the most abundant chemical classes, followed by terpenes, furans, ketones, and phenols. Most of these compounds are generated during the cooking, fermenting, and aging processes. In spite of the large similarities among beverages, the large differences in concentrations found can be used to identify the type of beverage under this study.

Sotols were the most complex alcoholic beverages among the studied in this work. There was the same number of esters in all beverages but short chain esters were more abundant in Sotol and long chain esters in Tequila and Mezcal. On the other hand, many alcohols were present in all samples and similarly to the esters, long chain alcohols were characteristic of Tequila and Mezcal, and short chain alcohols were mainly present in Sotol. Phenylethanol, a very

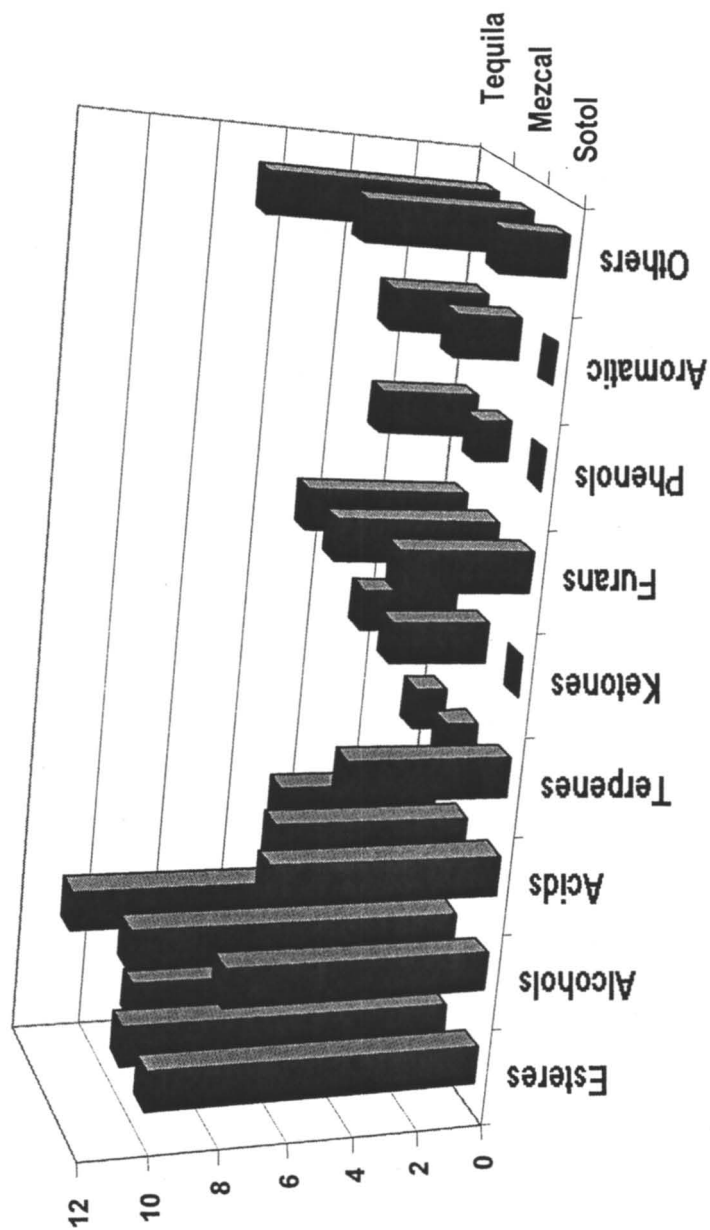


Figure 3. Main chemical groups found in Mexican spirits Sotol, Mezcal, and Tequila.

common volatile in many alcoholic beverages, was found in all samples, but the amount present in sotol was lower than the Mezcal and Tequila (592, 3713, and 3795, respectively). These criteria can also be used to discriminate among these beverages.

Many of the compounds found in Mezcal and Tequila have been previously identified using different extraction methods. But, this is the first time that Sotol volatiles have been reported.

Finally, it can be said that terpenes are the unique volatiles to authenticate a Tequila. Thirteen compounds were common to Sotol and Mezcal, four to Mezcal and Tequila, and only one between Sotol and Tequila. The presence of these compounds are useful to discriminate and establish origin and authenticity among Mexican alcoholic beverages.

Authenticity of Tequilas by Stable Isotopes

The adulteration of alcoholic beverages is an unfortunate practice due to the potential of high economic benefits. Therefore authenticity of the product, such as Tequila, are not only evaluated with origin denomination, but also with quality in ingredients used to make the adulterated product. Most modifications usually have cheaper and unsafe substituents, such as alcohols from other sugar sources, even methanol.

A Tequila process was monitored step by step up to the final product, during an actual Tequila production of about 100 tons of Agave pines. Stable isotope analyses of ethanol in Tequila were carried out to establish for the first time the ranges of delta values of important isotopes, such as $^{13/12}\text{C}_{\text{PDB}}$ (PDB = Pee Dee Belemnite) and $^{18/16}\text{O}_{\text{SMOW}}$ (SMOV = Standard mean ocean water). This study was performed in Blanco, reposado, and Añejo Tequilas, and in both categories, 100% and mixed types. Some $^2\text{H}_{\text{SMOW}}$ values have also been determined (data not shown). Isotopic data (delta values for ethanol) obtained from the analyses of the mentioned Tequilas of different brands and types (9) showed that delta values might be used to establish the authenticity of this alcoholic product. In addition, ten commercial samples were also included in the study. Table 2 lists some of the $\delta^{13}\text{C}_{\text{PDB}}$ and $\delta^{18}\text{O}_{\text{SMOW}}$ values found for Tequilas. The range values for carbon between -12.3 to -14.8 and for oxygen between 17.9 to 22.7 all are within the values corresponding to CAM plants.

Independently of the differences found in the $^{18}\text{O}_{\text{SMOW}}$ data between authentic and commercial samples, the $\delta^{13}\text{C}_{\text{PDB}}$ observed in all Tequilas fall within the range reported for CAM plants. A relevant difference was also found among 100% and mixed Tequilas (Figure 4). In spite of the data generated in this study, an evaluation of a larger number of Tequila samples as well as other Mexican distilled spirits made with different Agave species and their processes are necessary. This data is just the beginning of the creation of a Tequila data bank of stable isotopes.

Table II.- Delta values (δ) of $^{13}\text{C}_{\text{PDB}}$ and $^{18}\text{O}_{\text{SMOW}}$ of Tequila

Tequila	$^{13}\text{C}_{\text{PDB}}$	$^{18}\text{O}_{\text{SMOW}}$
Blanco 100%	-12.8	22.6
Blanco mixed	-14.8	21.2
Blanco commercial 100%	-12.9	19.0
Blanco commercial mixed	-13.3	18.1
Reposado 100%	-12.6	22.2
Reposado mixed	-12.5	21.3
Reposado 100%	-12.1	22.7
Reposado mixed	-13.9	21.7
Reposado 100%	-13.1	22.8
Reposado mixed	-13.9	20.8
Reposado 100%	-13.2	22.4
Reposado mixed	-13.9	21.1
Reposado commercial 100%	-12.7	22.7
Reposado commercial mixed	-13.8	19.0
Añejo 100%	-12.6	22.7
Añejo mixed	-12.7	21.5
Añejo 100%	-12.5	22.1
Añejo mixed	-13.4	21.2
Añejo commercial 100%	-12.5	21.3
Añejo commercial mixed	-12.3	17.9

Stable Isotopes of Tequila vs. *Agave tequilana* Weber azul variety

Delta values (δ) of carbon and oxygen uptake of a plant have been used as biomarkers for the establishment of authenticity, origin, and metabolic turnovers of a wide range of food products. Since many Mexican spirits are made with different *Agave* species, and most of which are CAM plants, it was very important to know if there were significant differences among the delta values (δ) of the raw materials used to elaborate the different spirits. Since the structure of *Agave* fructans have been published (16), it is now possible to determine the delta values of these Agavins and correlate them with isotopic data of Tequilas. The determination of $\delta^{13}\text{C}_{\text{PDB}}$ and $\delta^{18}\text{O}_{\text{SMOW}}$ in *Agave* species were performed (12). The observed differences on the $\delta^{13}\text{C}_{\text{PDB}}$ of different Agavins extracted from different *Agave* species of different geographic areas in Mexico (data not shown) may help to establish a direct relationship between

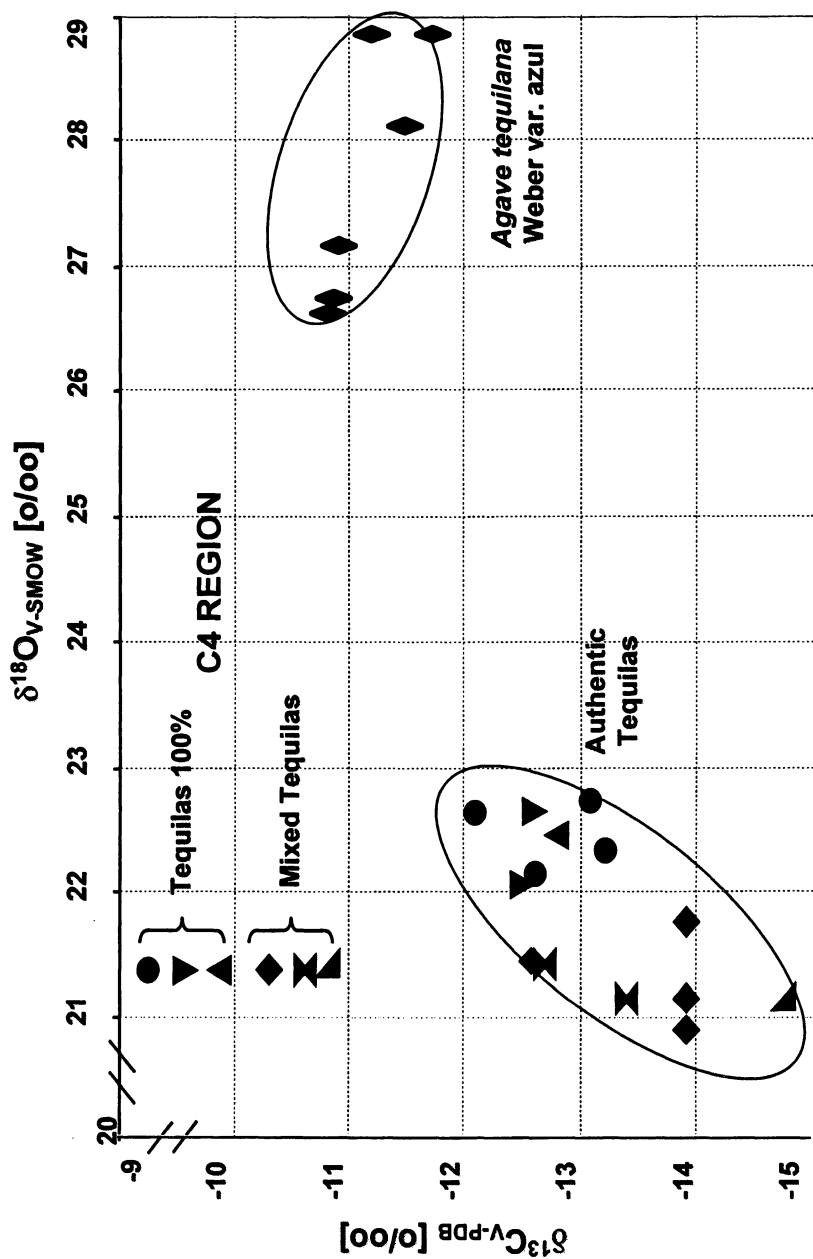


Figure 4. Delta values of ethanol from Tequilas and Agavins from *Agave tequilana* Weber azul variety.

the ethanol in a beverage and the sugar source (Agave species). It was also possible to establish the type of fractionation from the raw material (Agavins) (13) and the ethanol in authentic Tequilas, shown in Figure 4. The maximum discrimination was observed in both, $\delta^{13}\text{C}_{\text{PDB}}$ and $\delta^{18}\text{O}_{\text{SMOW}}$. ^{13}C values of Tequilas were more negative (larger amount of ^{12}C), but this change was larger for mixed Tequilas. The more enriched delta values of ^{18}O in Agavins denote the real value of the Agave fructans, because the ^{18}O values in the Tequilas have been influenced by the water used to standardized the alcoholic degree of the beverages. More samples are required to build a database in order to distinguish the *Agave tequilana* Weber azul variety.

Multivariate Analysis of Tequilas by FTIR

Another powerful analytical technique to investigate the authenticity of a product is FTIR (Fourier Transformed Infrared Spectroscopy). This technique can be potentially used to certify and control the authenticity of Tequilas at low cost, less analysis time, and almost no sample manipulation. Lachenmeier et al., (2005) reported for the first time the classification of authentic and commercial Tequilas in both categories (100% and mixed tequilas), using FTIR along with multivariate analysis. Figure 5 shows a separation of different Tequilas and 100% authentic and mixed products. However, commercial tequilas presented the largest differences among all, not forming part of either the 100% or mixed types of the authentic samples.

Perspectives

Despite all the research performed in Tequila using many different analytical techniques, there is still more information needed such as:

1. Analysis of more authentic Tequila from different companies as well as their processes.
2. Keep looking for a biomarker compound that can be followed from the raw material to the final product.
3. Increase database (library) for stable isotope values of both Agave species and Tequila.
4. Create a database using a non-destructible and low cost technique such as FTIR/NIR.
5. Generate intra- and inter-lab analysis.

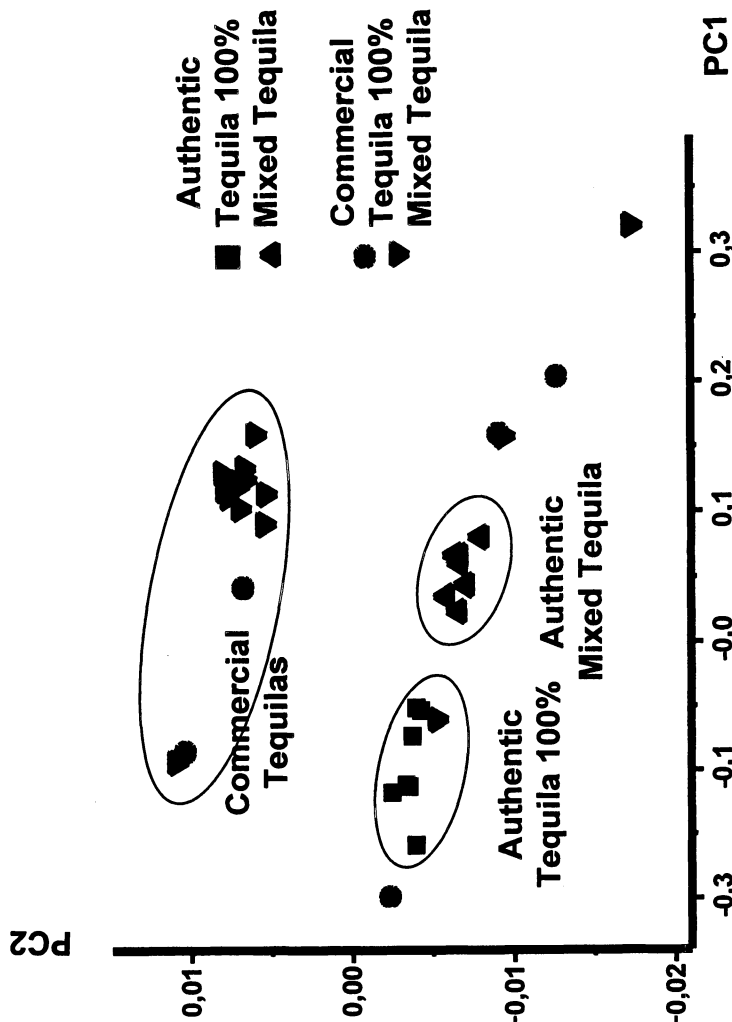


Figure 5. PCA of de complete FTIR of 38 Tequila samples.

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Chapter 19

Identification of Green Tea (*Camellia sinensis* L.) and Tea Oil (*Camellia oleifera* Abel.) by Molecular, Biological, and Anatomical Methods

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Green tea is associated with numerous health-related benefits, including amelioration of cancer and enhancement of insulin activity. The quality of many botanicals and dietary supplements, relies primarily on “Good Manufacturing Practices”. However, plants are frequently misidentified or intentionally or unintentionally contaminated in the preparation of botanicals. We developed molecular biological approaches to verify the identity of plant material in dietary supplements and botanicals. In this report, we analyzed DNA isolated from living tea plants as well as from a close relative, the tea oil plant, using both molecular and anatomical methods. We then examined DNA isolated from dried green tea from commercial sources, and authenticated that the tested commercial products contained tea.

Green tea is one of the most popular beverages in the U.S. market, not only for its perceived health benefits, but also for its taste and stimulant effects. Green tea is rich in flavonoids, especially flavan-3-ols, which have antioxidant, anti-carcinogenic, anti-allergenic, anti-inflammatory, and vasodilatory properties (1). Polyphenolics comprise 30–40% of the extractable solids of dried green tea leaves, with the catechins being of primary importance as possible preventative agents against cancer (2). Moreover, catechins and polysaccharides, which are also found in green tea, have been shown to have anti-diabetic activity (3).

In spite of the benefits of green tea, the use of this plant and other herbal supplements for health purposes is largely unregulated by the Food and Drug Administration. The Dietary Supplement Health and Education Act (DSHEA), passed by Congress in 1994, allowed the establishment of Good Manufacturing Practices (GMPs) to help assure a product's quality. In 2003, the FDA approved new criteria, i.e. "Current Good Manufacturing Practices" (CGMPs), towards developing industry-wide standards for botanicals and dietary supplements. However, current methods to verify the authenticity of these products and to detect any potentially harmful contaminants are based mainly on taxonomic characterization, which often requires considerable expertise in analyzing macroscopic and microscopic features, or on phytochemical parameters, which may be variable depending on how the plants were grown or how the original plant materials were processed.

Recently, DNA-based methods have been used to give a more definitive verification of plant material found in herbal dietary supplements and botanicals. We have used these tools and developed additional molecular methods to isolate and if needed, repair degraded DNA, isolated from botanicals, as part of the authentication process (4). In addition, we have developed strategies to identify the components of mixed samples with the goal of detecting contaminants, adulterants, or mislabeled plant material in botanicals (5).

With these tools in hand, we pursued an investigation of green tea from commercial sources. We first established a base line by analyzing living specimens of *Camellia sinensis* L., the source of green, oolong, and black tea, and another species in the genus, *Camellia oleifera* Abel., the source of tea oil, which has been utilized for cooking oil, cosmetics, and livestock feed. We amplified and sequenced DNA of the ITS (internal transcribed spacer) region of the nuclear ribosomal DNA gene, and of *matK*, an intron in the chloroplast gene *trnK*. The living tea plants and two commercial green tea products were each found to contain multiple ITS sequences, which showed the greatest similarity to *C. sinensis* or other *Camellia* species. Because no ITS sequences for *C. oleifera* have been deposited in Genbank, the young tea oil plants were subjected to a taxonomic analysis, based on vegetative characters, to confirm their identity. Several ITS products of variable length and sequence were also obtained from DNA of the tea oil plant. The greatest similarity of these DNA sequences was to species of *Camellia*, as well as to two other members of the Theaceae, *Pyrenaria*

and *Tutcheria*. DNA sequencing of *matK* identified the tea plants and two commercial green tea products as *Camellia sinensis*, and additionally revealed a one-nucleotide difference between the tea and tea oil DNA sequences.

Materials and Methods

Plant Material

Living plants of *Camellia sinensis* L. (tea) and *C. oleifera* Abel. (tea oil) were purchased from Camellia Forest Nursery in Chapel Hill, NC, and subsequently grown in the UCLA Plant Growth Center (PGC), or were grown outside in the UCLA Herb Garden. Two commercial products containing green tea were purchased from local supermarkets and pharmacies.

DNA isolation

Leaves were collected from living specimens, and dried tissue was obtained from the commercial products. Approximately 100 mg of fresh tissue and 40 mg of dried tissue were ground under liquid nitrogen using a mortar and pestle. DNA was isolated using the Qiagen Plant DNeasy Kit (Qiagen, Valencia, CA). The DNA was quantified spectrophotometrically and also on 1% agarose gels to check whether or not the DNA was degraded. If degraded, the DNA was subjected to a two-step repair reaction as described in (4).

PCR of DNA and Cloning

A polymerase chain reaction (PCR) was performed using the ITS universal primers, A and B, to amplify the ITS region (6). *matK* was amplified using the forward primer 5'-TATGSACTTGGTYATRRTCAT-3' and the reverse primer 5'-GAACYAAXATTTCCARATGGA-3'. Reactions were carried out in a final volume of 25 μ L following the procedure outlined in (5). In PCR-amplifications using a proofreading polymerase, the reactions were performed using 200 μ M dNTPs, 1x PCR buffer, 3 mM MgSO₄, 2% DMSO, 1 μ M of each primer and 1 U Deep Vent DNA Polymerase (New England Biolabs Inc., Ipswich, MA) and employing a 3-minute instead of a 30-second elongation step for each cycle. The PCR products were subjected to electrophoresis on a 1% agarose gel. DNA from the PCR-amplified bands was extracted using the Qiagen Gel Extraction Kit for

use in direct sequencing. The extracted DNA was incubated with 0.5 U Taq DNA Polymerase (Invitrogen, Carlsbad, CA), 1x PCR buffer and 200 μ M dATP for 10 minutes at 72°C to add a 3' adenosine overhang to products initially amplified by a proofreading polymerase. Two μ L of the reaction was directly used for TA cloning using the Invitrogen TOPO TA Cloning Kit for Sequencing.

Sequencing Reactions and Sequence Analysis

Sequencing was performed using ABI Big Dye Terminator mix and run at the UCLA Sequencing facility. The PCR products were sequenced in both directions using the forward or reverse ITS or *matK* primers. The T3 or T7 primers were used to sequence the TOPO clones in each direction.

The DNA sequences were analyzed using BLAST (7). The sequence alignments were performed using CLUSTAL W 1.8 (8) with output produced by Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Percent identity was calculated using Bestfit (Wisconsin Package Version 10.3, Accelrys Inc., San Diego, CA). ITS sequences obtained from Genbank and used in the analysis are AF315492 (*C. sinensis*; Csinensis_GB1) and AY096014 (*C. sinensis*; Csinensis_GB2). The *C. sinensis matK* gene is accession AF380077.

Analysis of Taxonomic Characters

Using the treatments of Theaceae from several floras (9, 10, 11), the following suite of vegetative characters was used to analyze two plants purchased and labeled as *C. oleifera*:

- Petiole vesture and length;
- Leaf dimensions, shape, margin;
- Leaf midvein vesture and prominence (abaxially and adaxially);
- Leaf secondary veins (number per side and prominence);
- First year branch color and vesture.

The living *C. oleifera* plants were compared with the descriptions of 106 species of *Camellia*, six *Tutcheria* species, and one *Polyspora* species.

Results

Analysis of living specimens of *C. sinensis* and *C. oleifera*

High molecular weight genomic DNA was isolated from two different plants of each *C. sinensis* (Green tea) and *C. oleifera* (Tea oil) (Fig. 1A, data not

shown). Amplification of the ITS region resulted in multiple bands of varying sizes and intensities in preparations from both species (Fig. 1B). Direct sequencing of the bands of the correct size for the ITS in both cases gave ambiguous results with many uncalled bases, which suggested that variable ITS sequences might be present, or alternatively, that DNA sequences other than the ITS region were amplified.

Because of concerns that the variability we detected may be in part due to Taq polymerase error and/or recombination occurring between different alleles during PCR (12), we used a proofreading polymerase combined with an increased extension time (12). The appropriately sized PCR products were then cloned and sequenced. Multiple ITS sequences were obtained, two of which are represented in Figure 2. One of these sequences (Greentea1) showed 99.2% identity to one of the *C. sinensis* sequences in the database (Csinensis_GB1, Fig. 2). In contrast, a second sequence (Greentea2), although most similar to *C. sinensis*, only showed 91.2% identity (Csinensis_GB1, Fig. 2). However, this sequence was identified in our analysis of a second green tea plant with an identity of 98.5%.

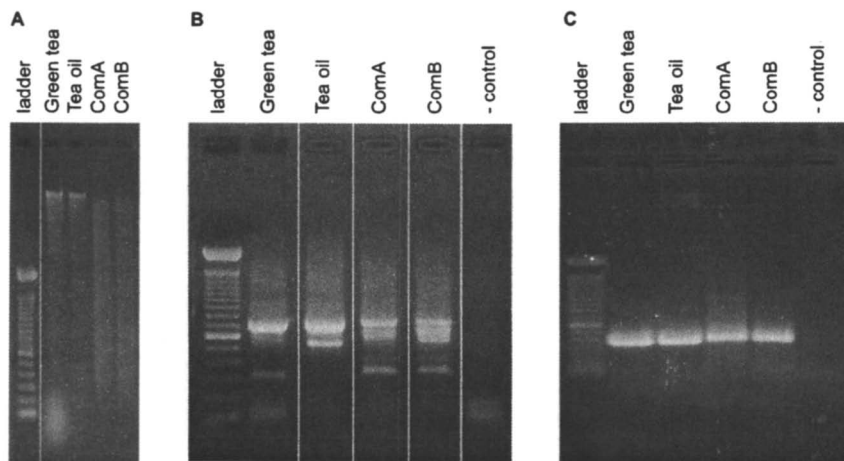


Figure 1. Agarose gel electrophoresis showed that high molecular weight DNA was obtained from living specimens of Green tea and Tea oil, but that DNA from commercial products (ComA, ComB) was degraded (A). Multiple ITS PCR products were obtained from (B) Green tea, Tea oil, and the commercial products. In contrast, there was only a single *matK* band for all samples (C).

Two dominant bands, presumably ITS products of variable length, were obtained from the tea oil specimen (Fig. 1B). Analysis of five clones from each plant showed variability in the sequences (data not shown). A representative sequence from plant 1 that was 99.4% identical to a sequence in the second tea oil specimen had greatest similarity to the ITS region from species of *Camellia* (89.7%). Other sequences showed a high similarity to the ITS from species of *Tucheria* and *Polyspora* (data not shown).

An analysis of a 347-nucleotide region of the *matK* gene was also performed on the DNA isolated from the tea and tea oil specimens and revealed a single PCR product (Fig. 1C). Sequence analysis showed each was most similar to the *C. sinensis matK* sequence in Genbank; no *C. oleifera matK* sequence has been deposited in the database. The *matK* fragment from the *C. sinensis* specimen was identical to that of *C. sinensis* in Genbank as well as to two other *Camellia* species and also to *Glyptocarpa camellioides*. The *matK* DNA sequence from the *C. oleifera* specimen was also most similar to *C. sinensis matK* in Genbank, but had a single nucleotide substitution (Fig. 3).

To determine whether or not the *C. oleifera* plants we obtained were correctly labeled, a taxonomic evaluation of the vegetative traits, using the parameters outlined in the Materials and Methods, was performed. Table 1 lists the characters of the reference *C. oleifera*, from (9, 10, 11), and the comparable traits from the two plants growing in the UCLA Herb Garden. All the characters for plants 1 and 2 match or are within the range of values for the characters of *C. oleifera*, except for some minor variation. Some of the variation may be attributed to the fact that the measurements made of the reference specimen are from dried leaves whereas the comparable measurements from plants 1 and 2 are from fresh leaves.

Plants 1 and 2 were also compared with other species of *Camellia* that are similar, but were excluded for the reasons listed: *C. cupiformis* (petiole too short, first year branch color grayish yellow), *C. kissei* (first year branch color grayish, secondary veins abaxially prominent), *C. szemaoensis* (first year branch color grayish yellow, pubescent, secondary veins prominent), *C. caudata* (first year branch vesture villose and pubescent, secondary veins abaxially prominent), and *C. synaptica* (first year branch grayish yellowish, pubescent, petioles too short).

Comparison of plants 1 and 2 to other species that had similar nucleic acid base sequences for the ITS region (*T. maculatoclada*, *T. greeniae*, *Polyspora kwangsiensis* (Hung T. Chang) C.X.Ye, *T. sophiae* (Hu) Hung T. Chang, *T. pingpienensis* Hung T. Chang, *T. spectabilis* (Champion) Dunn, and *T. microcarpa* Dunn) revealed that they differed from *C. oleifera* in 3 to 5 characters (Table 2). Thus, we conclude that the two plants were correctly labeled as *C. oleifera*, and we have submitted the DNA sequences for the ITS region of *C. oleifera* to Genbank.

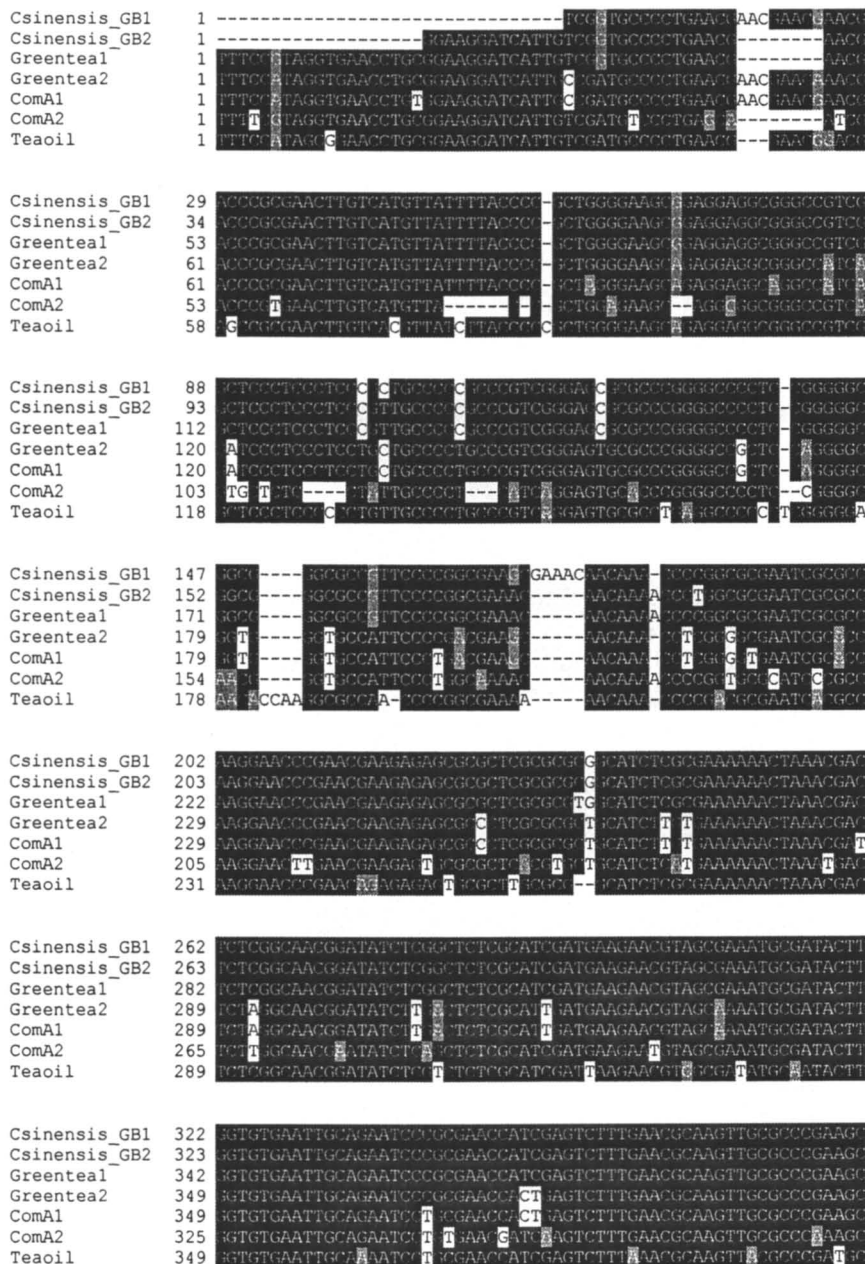


Figure 2. Comparison of representative ITS sequences obtained from the living specimens of green tea (*Greentea1,2*), tea oil (*Teaoil*), and one of the commercial products (*ComA1,2*). These sequences showed a high degree of variability among themselves and to the green tea sequences in the database (*Csinensis_GB1,2*).


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Csinensis_GB1 382  ATTAGGTTGAGGGCACCTTGCTGGGGCTCTCACGTTGGCTCGCCCGCGCCCTCCG
Csinensis_GB2 383  ATTAGGTTGAGGGCACCTTGCTGGGGCTCTCACGTTGGCTCGCCCGCGCCCTCCG
Greenteal 402  ATTAGGTTGAGGGCACCTTGCTGGGGCTCTCACGTTGGCTCGCCCGCGCCCTCCG
Greentea2 409  ATTAGGTTGAGGGCACGTCCTCTGGGCTCTCACGTTGGCTCGCCCGCGCTCCG
ComA1 409  ATTAGGTTGAGGGCACGTCCTCTGGGCTCTCACGTTGGCTCGCCCGCGCTCCG
ComA2 385  ATTAGGTTGAGGACCTGCTGCTGGGCTCTCACGTTGGCTCGCCCGCTCTCTAG
Teaoil 409  ATTAGGTTGAGGGCACCTTGCTGGGGCTCTCACGTTGGCTCGCCCGCGCTCCG

Csinensis_GB1 442  CTTGCGAAAATGAAGCGGGGAAGAAGGGCTGCGGGCGGATGTTGGCCCGTTCGCGG
Csinensis_GB2 443  CTTGCG-----GAAGCGGGGAAGAAGGGCTGCGGGCGGATGTTGGCCCGTTCGCGG
Greenteal 462  CTTGCG-----GAAGCGGGGAAGAAGGGCTGCGGGCGGATGTTGGCCCGTTCGCGG
Greentea2 469  TTT-----GAAGCGGGGAAGAAGGGCTGCGGGCGGATGTTGGCCCGTTCGCTTC
ComA1 469  TTT-----GAAGCGGGGAAGAAGGGCTGCGGGCGGATGTTGGCCCGTTCGCTTC
ComA2 445  TTTA-----GAAGCGGGGAAGAAGGGCTGCGGGCGGATGTTGGCGTATGTTTC
Teaoil 469  TTTCCCCCCCCCTTTTCTTGGGAAGGAGGGCTGCTCTGGATGTTGGCCCGTTCGCTTC

Csinensis_GB1 500  TGTGGCGCGGTCGGCCAAAAGCGAGTCCCGCGGACATGACGCGTCGGGAGAGT
Csinensis_GB2 494  TCCCGCGCGCGGTCGGCCAAAAGCGAGTCCCGCGGACATGACGCGTCGGGAGAGT
Greenteal 513  TCCCGCGCGCGGTCGGCCAAAAGCGAGTCCCGCGGACATGACGCGTCGGGAGAGT
Greentea2 518  TCCCGCGCGGCTGCGGCTTAAAATGAGTCCCGTTCGATGACGACATGCGGAGAGT
ComA1 517  TCCCGTTCGATGTCGGCTTAAAATGAGTCCCGTTCGATGACGACATGCGGAGAGT
ComA2 490  TCTGCGCGCGGTTCTCTTAAAATGAGTCCCGTTCGATGACGACATGCGGAGAGT
Teaoil 527  TCTTTTCTGCGGTTGCGCCAAAATGAGTCCCGTTCGATGACGACGCGTCGGGATGAGT

Csinensis_GB1 560  GTGGTTGACAAACCGTTGCTGCGCTCGCGCGCGTCCGTTGTCGCGGGAGGCTGTG
Csinensis_GB2 554  GTGGTTGACAAACCGTTGCTGCGCTCGCGCGCGTCCGTTGTCGCGGGAGGCGTGTG
Greenteal 573  GTGGTTGACAAACCGTTGCTGCGCTCGCGCGCGTCCGTTGTCGCGGGAGGCGTGTG
Greentea2 577  GTGGTTGACAAACCGTTGCTGCGCTCGCGCGCGTCCGTTGTCGCGGGAGGCGTGTG
ComA1 576  GTGGTTGACAAACCGTTGCTGCGCTCGCGCGCGTCCGTTGTCGCGGGAGGCGTGTG
ComA2 550  GTGGTTGACAAACCGTTGCTGCGCTCGCGCGCGTCCGTTGTCGCGGGAGGCGTGTG
Teaoil 586  GTGGTTGACAAACCGTTGCTGCGCTCGCGCGCGTCCGTTGTCGCGGGAGGCGTGTG

Csinensis_GB1 620  GTGACCCATATCGGCGCGCCCAAGG-----CTCCGA-----
Csinensis_GB2 614  GTGACCCATATCGGCGCGCCCAAGG-----CTCCGATCGGACCTTAT-----
Greenteal 633  GTGACCCATATCGGCGCGCCCAAGG-----CTCCGATCGGACCCAGGTGAGG
Greentea2 637  GTGACCCATATCGGCGCGCCCAAGG-----CTCCGATCGGACCCAGGTGAGG
ComA1 636  GTGACCCATATCGGCGCGCCCAAGG-----CTCCGATCGGACCCAGGTGAGG
ComA2 610  GTGACCCATATCGGCGCTTCCCAAGG-----CTCTTATCGGACCCAGGTGAGG
Teaoil 646  GTGACCCATATCGGCTTCCCAAGGCAAGGTCCTTCTCTGACCCCTTGTGTCAGG

Csinensis_GB1 -----
Csinensis_GB2 -----
Greenteal 688  NTTACCGCTGAGTTTAA
Greentea2 692  ATTACCGCTGAGTTTAA
ComA1 691  ATTATCTGAGTTTAA
ComA2 665  ATTACCTGAGTTTAA
Teaoil 706  CSTTACCGCTGAGTTTAA

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Figure 2. Continued.

Greentea	1	ATGGGTTAACTAGATCGATTTTTGGGAAAATATAGGTTATGACAATAAAATTCAGCTTA
Com	1	ATGGGTTAACTAGATCGATTTTTGGGAAAATATAGGTTATGACAATAAAATTCAGCTTA
Teaoil	1	ATGGGTTAACTAGATCGATTTTTGGGAAAATATAGGTTATGACAATAAAATTCAGCTTA
Csinensis_GB	1	ATGGGTTAACTAGATCGATTTTTGGGAAAATATAGGTTATGACAATAAAATTCAGCTTA
Greentea	61	TAATTGTGAAACATTTAATTACTCAAATGTATCAACAGAATCATTTCCTTTTCTGCT
Com	61	TAATTGTGAAACATTTAATTACTCAAATGTATCAACAGAATCATTTCCTTTTCTGCT
Teaoil	61	TAATTGTGAAACATTTAATTACTCAAATGATCAACAGAATCATTTCCTTTTCTGCT
Csinensis_GB	61	TAATTGTGAAACATTTAATTACTCAAATGTATCAACAGAATCATTTCCTTTTCTGCT
Greentea	121	AATGATTCCTAACCAAAATCCATTTTGGGCACACACGAAATTTGTATTCTCAAATGATA
Com	121	AATGATTCCTAACCAAAATCCATTTTGGGCACACACGAAATTTGTATTCTCAAATGATA
Teaoil	121	AATGATTCCTAACCAAAATCCATTTTGGGCACACACGAAATTTGTATTCTCAAATGATA
Csinensis_GB	121	AATGATTCCTAACCAAAATCCATTTTGGGCACACACGAAATTTGTATTCTCAAATGATA
Greentea	181	TTAGAAGGATTTGTAGCCGTTGTGGAAATTCATTTTCTCTATTTCCCTAGAAGGTAAAT
Com	181	TTAGAAGGATTTGTAGCCGTTGTGGAAATTCATTTTCTCTATTTCCCTAGAAGGTAAAT
Teaoil	181	TTAGAAGGATTTGTAGCCGTTGTGGAAATTCATTTTCTCTATTTCCCTAGAAGGTAAAT
Csinensis_GB	181	TTAGAAGGATTTGTAGCCGTTGTGGAAATTCATTTTCTCTATTTCCCTAGAAGGTAAAT
Greentea	241	GAATAGTGAAATCTCAAAATTTACGATCAATTCATTCAATATTTCCCTTTTGTAGAGGAC
Com	241	GAATAGTGAAATCTCAAAATTTACGATCAATTCATTCAATATTTCCCTTTTGTAGAGGAC
Teaoil	241	GAATAGTGAAATCTCAAAATTTACGATCAATTCATTCAATATTTCCCTTTTGTAGAGGAC
Csinensis_GB	241	GAATAGTGAAATCTCAAAATTTACGATCAATTCATTCAATATTTCCCTTTTGTAGAGGAC
Greentea	301	AAATTTTCACATCTAAATTTATGTGTTAGATATACTAATACCCCACTCCATCCATCTG
Com	301	AAATTTTCACATCTAAATTTATGTGTTAGATATACTAATACCCCACTCCATCCATCTG
Teaoil	301	AAATTTTCACATCTAAATTTATGTGTTAGATATACTAATACCCCACTCCATCCATCTG
Csinensis_GB	301	AAATTTTCACATCTAAATTTATGTGTTAGATATACTAATACCCCACTCCATCCATCTG

Figure 3. The top match from the database for the matK DNA sequence from the living specimens of tea (*Greentea*) and tea oil (*Teaoil*) was *C. sinensis* (*Csinensis_GB*). Compared to the matK sequence obtained from the living tea specimen, the commercial green tea products (*Com*) were identical and the tea oil DNA sequence had a one-nucleotide difference.

Table 1. Comparison of the reference *C. oleifera* with plants 1 and 2.

<i>Characters</i>	<i>Camellia oleifera</i>	<i>Plant 1</i>	<i>Plant 2</i>
First yr stem			
color	reddish brown		reddish brown
vesture	pubescent		sparsely ascending pubescence
Petiole			
length (mm)	5--10	5--6	5--9
vesture	pubescent	adaxially pubescent	adaxially and abaxially pubescent
Leaf blade			
length (cm)	3--12	6--7.5	5--10
width (cm)	2--5	2.5--3.5	2--6
margin	serrate to serrulate		serrulate
vesture			
adaxial surface	glabrous		glabrous
abaxial surface	glabrous		glabrous
Midvein			
adaxial vesture	hirtellous		hirtellous
abaxial vesture	pilose to glabrous	glabrous	glabrous or hirtellous proximally
abaxial prominence	prominent		raised and prominent
abaxial prominence	prominent		+/- prominent
Secondary veins			
number per side	5--8	7--9	5--7
abaxial prominence	prominent		obscure but becoming slightly prominent on drying
abaxial prominence	obscure		obscure

Table 2. Characters that differ from *C. oleifera*

Species	First year stem vestiture	Petiole vestiture	Midvein adaxial vestiture	Mitvein adaxil prominence	Secondary vein number	Abaxial vein prominence
<i>Tutcheria maculatochlada</i> (Pyrenaria maculatochlada *)	glabrous	glabrous	glabrous	impressed		
<i>T. greeniae</i> (Pyrenaria spectabilis var. greeniae *)	yellowish or grayish brown		glabrous	impressed	12-16	prominent
<i>Polyspora kwangsiensis</i> (Polyspora acuminata *)	glabrous	glabrous	glabrous	impressed		
<i>T. sophiae</i> (Pyrenaria sophiae *)	pale yellow			impressed		prominent
<i>T. pingpienensis</i> (Pyrenaria pingpienensis *)	glabrous		glabrous	impressed	8-12	
<i>T. kweichowensis</i> (see <i>T. pingpienensis</i>)						
<i>T. microcarpa</i> (Pyrenaria microcarpa *)	yellowish brown			impressed	8-12	

*Name used in (6)

A similar taxonomic analysis was made of the *C. sinensis* plants growing in the UCLA Herb Garden (data not shown). Again, we concluded that these plants had been correctly labeled as *C. sinensis*.

Analysis of a green tea commercial product

DNA obtained from the two different commercial products was degraded (Fig. 1A) and had to be repaired before a PCR product could be obtained. The ITS region was amplified and multiple bands were revealed on an agarose gel (Fig. 1B). Cloning and sequencing of each of the bands corresponding to the predicted size of the ITS region revealed that there was a high degree of variability of the sequences for both commercial products (Fig. 2, ComA1, 2; data not shown). From the first commercial product, one of the sequences showed the greatest similarity to one of the *C. sinensis* sequences we had obtained (98.2%) (ComA1 vs. Greentea2). However, it was less than 91% identical to the other green tea sequence we obtained and to the sequences in the database. A second sequence, identified also in the second commercial product (99% identity) showed greater identity to *C. japonicum* (89.0%) than to *C. sinensis* (85.8%, Greentea1).

Discussion

We have used molecular methods based on amplifying and sequencing the ITS region of nuclear ribosomal RNA and *matK*, an intron in the chloroplast gene *trnK*, to authenticate living specimens of *C. sinensis* and two commercial products sold as green tea. Previously, Random Amplified Polymorphic DNA (RAPD) markers (13) and Sequence Tagged Site-Restriction Fragment Length Polymorphism (STS-RFLP) analysis (14) have been used to examine genetic diversity in tea as well as to identify processed Japanese green tea. The latter studies elegantly showed the differences in DNA banding patterns among a diversity of tea genotypes.

The ITS region is frequently used for phylogenetic analyses because it is a moderately to slowly evolving DNA sequence and because it is a nuclear-encoded gene (13). Moreover, it is found in high copy numbers, which makes it easy to detect by PCR. Typically there is only a single allele present in a population due to rapid concerted evolution (15). However, variations can exist in the DNA sequence of the different copies of the ITS region; most commonly, point mutations, and more infrequently, length mutations caused by insertions/deletions (indels) (16). There have been a number of reports of multiple variable rDNA sequences within individuals (17), some of which may be pseudogenes (18).

The variability observed in the *C. sinensis* ITS DNA when sequenced directly led us to clone the PCR-amplified DNA and then sequence individual clones. In so doing, we found a 99.2% match of a sequence from the green tea plant to that of *C. sinensis* in the database. However, there were also other sequences represented that varied by greater than 8%, but which were highly conserved in both of the green tea plants we analyzed.

Based on our analysis of the sequences and the presence of two different *C. sinensis* ITS sequences in the database, we suspect that green tea may be a species that exhibits a high degree of variability. A number of reports have shown that *in vitro* recombination between similar templates can occur at a high frequency, leading to chimeric molecules, regardless of which DNA polymerase is used (see 17). With this in mind, we used a proofreading polymerase and extended the elongation time of the PCR reaction prior to cloning of the products. We have only presented those sequences that were present in multiple, independent samples. However, an overview of all the sequences we obtained shows that they group into distinct clusters. A more thorough analysis will be needed to determine how much variation is present.

To help resolve the identity issue, we utilized *matK* primers to amplify a fragment of chloroplast DNA. The *matK* gene encodes a maturase, which is involved in splicing type II introns from RNA transcripts and is a rapidly evolving gene in the chloroplast genome (19). The fact that the chloroplast genome, and consequently *matK*, is uniparentally inherited makes it unlikely that recombination will occur. We found that the *matK* sequence of the living green tea plant and the commercial products was identical to that of the *C. sinensis* Genbank entry. However, two other *Camellia* species and a *Glyptocarpa* species have the identical sequence, making the resolution of species in the Theaceae difficult. Previous studies using *rbcl* and *matK* show that these nucleotide sequences are not variable enough to address relationships within Theaceae (20). When we used *matK* for *C. oleifera* the closest sequence similarity was to the *C. sinensis matK* in the database, although a one-nucleotide substitution was present.

When the ITS region of *C. oleifera* was analyzed, two bands were visible on an agarose gel (Fig. 1B). Sequence analysis demonstrated that the higher molecular weight band had highest identity to species of *Camellia*. Additional sequences showed similarity to species of *Polyspora* and *Tucheria*, two members of Theaceae sensu stricto (20). An ITS sequence for *C. oleifera* has not been deposited yet in Genbank, so it is not surprising that the ITS region shows similarity to other members of the Theaceae, which have been sequenced and submitted to Genbank.

It will be interesting to determine if the polymorphic nature of the ITS region holds true for other *Camellia* species and the different cultivars of tea. Although the ITS region may not be a good marker for *Camellia* species identification, ITS polymorphism, if shown for other cultivars, could be a useful molecular method of detecting variation in different cultivars. Although we did

not examine the smaller bands in Fig. 1B in detail, preliminary analysis shows that these are also ITS, but they may be microbial contaminants based on sequence analysis (data not shown). We are pursuing this analysis further.

Anatomical and morphological characters are extremely valuable for deducing plant identity, so we analyzed a number of vegetative characters for the presumed *C. oleifera* plants. A detailed study of these traits showed that the plants showing the most similar DNA sequences, including *T. maculatochlada*, differed from the young tea oil plants in 3 to 5 characters, supporting the idea that the latter are indeed *C. oleifera*. Because vegetative characters may not always be definitive, we will review the floral characteristics of the plants when they mature.

In the past, analysis of anatomical and phytochemical characters was the only means to authenticate the identity of plant materials in botanicals and herbal dietary supplements. Analysis of selected DNA sequences, which can function as bar codes for plant identity, are an additional useful tool. However, as clearly shown here, when a DNA sequence is not available in the database for comparison or is highly variable, other methods of verifying authenticity are required. Ultimately, the combination of molecular, phytochemical, and anatomical procedures will lead to the verification and authentication of a wide variety of botanicals and dietary supplements, thereby ensuring the safety of people who consume them.

Acknowledgments

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Chapter 20

Health Food and Medicine: Combined Chemical and Molecular Technologies for Authentication and Quality Control

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Many plants and animals and their products are being used as medicine or health food. To protect consumer rights and ensure the pharmaceutical value, it is essential to establish effective methods for authentication and quality control. This review introduces the common chemical and molecular technologies for species identification and quality control. The former includes techniques in chromatography, spectrometry and combined methods. The latter includes DNA fingerprinting and sequencing. Examples of each technique are given and their applications and limitations are evaluated.

Introduction

Humans use plants and animals as medicine for the treatment of various chronic and infectious diseases for thousands of years. For example, traditional Chinese medicine has been extensively documented with the first record being Wu-Shi-Er-Bing-Fang compiled between 1065 and 771 BC containing 283 prescriptions, which was followed by others such as Sheng-Nong-Ben-Cao-Jing (~200 AD, 365 drugs), Xin-Xiu-Ben-Cao (659 AD, Tang dynasty, 850 drugs), Tu-Jing-Ben-Cao (1062 AD, Song dynasty, 1082 drugs) and Ben-Cao-Gang-Mu (1593 AD, Ming dynasty, 1892 drugs) (1).

Nowadays, plant and animal materials continue to play an important role in health care, especially in Asian countries. It has been estimated by the World Health Organization that approximately 80% of the world's population are using traditional medicine for primary health care (2). Moreover, more and more functional food or dietary supplements, mainly of plant origin, appear in recent years. They serve as an auxiliary support for preventing and treating health disorder.

With the increasing popularity of herbal medicine and dietary supplements, quality control has become an important issue (3). The botanical resource is the primary factor influencing the quality. To have the correct pharmacological effect, it is essential to use the right herbal material. Life-threatening poisoning may occur if the intended herbs are erroneously substituted with toxic plants with similar morphology or name (4). In addition, the quality of herb is also influenced by the physiological conditions, place of collection, harvesting time, storage conditions, processing methods and manufacturing procedure.

A battery of techniques has been developed for the authentication and standardization of herbal medicine and dietary supplements in recent years. Among them, chemical technology is the main method used, because of its high efficacy in providing qualitative and quantitative assessment of the chemical composition of the material (5). On the other hand, molecular technology is powerful in elucidating the identity of the material and the relationship of closely related species. Combining the two technologies, it is possible to authenticate and assess the quality of the material to high accuracy. The following review provides a brief summary and evaluation of the major chemical and molecular techniques.

Chemical Technologies

Chemical Technologies for Quality Control

Most herbal medicine and functional food are plant crude extracts. Besides the large amount of protein, polysaccharides, resin and tannin (primary

metabolites), they usually contain up to hundreds or even thousands of different phytochemicals (secondary metabolites), which makes quality control extremely difficult. Therefore, various chromatographic and spectroscopic methods have been developed for the separation and identification of the bioactive principles.

Chromatography

Chromatography is the process of separating the components of mixtures that are distributed between a stationary phase and a flowing mobile phase according to the migration rate of individual molecules. Chromatographic techniques used for identification and analysis mainly include thin layer chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), supercritical-fluid chromatography (SFC) and capillary electrophoresis (CE). In the Chinese Pharmacopoeia, TLC, HPLC, GC and CE are included as the official methods for the quality control of some medicinal herbs (6).

Thin Layer Chromatography (TLC)

TLC remains a common analytical method with advantages of low cost and easy operation. It is primarily used as a qualitative method for the identification of chemicals by comparison of samples with the reference standards. For example, TLC method has been used for the comparison of the constituents of American and Chinese ginseng. 24(R)-pseudo-ginsenoside-F11 was found to be the characteristic constituent of American ginseng and ginsenoside-RF was that of Chinese ginseng (7). Besides its application in herbal medicine, TLC was also widely used for the analysis of agricultural products, foods and beverages (8).

High Performance Liquid Chromatography (HPLC)

HPLC is also a commonly used technique. It can provide both qualitative and quantitative surveys of the composition in the extract. Reverse phase HPLC was used to compare Cassia bark, a popular natural spice, with its adulterants. The results showed that cassia bark contained high contents of cinnamaldehyde (13.01-56.93 mg/g). In contrast, the adulterants from the other *Cinnamomum* species contained low contents of cinnamaldehyde (<2.00 mg/g). An HPLC fingerprint comprised of five characteristic peaks was established for the quality control of this commodity (9). HPLC profiles were used for the comparison of the four species of *Gentiana* used as *Radix Gentianae* and differentiation from

those used as adulterants. The close similarity of chemical compositions among the four genuine *Gentiana* species explains their popular usage as Radix Gentianae in Chinese medicine. It was shown that the variation of chemical composition in Radix Gentianae and related adulterants agree well with their botanical phylogeny (10). A reversed-phase high-performance liquid chromatography method was developed for the simultaneous determination of diosmin, hesperidin, and naringin in different citrus fruit juices. All three flavonoids and rhoifolin (internal standard) were separated using tetrahydrofuran/water/acetic acid (21:77:2) as the mobile phase at 34°C using a C8 reversed-phase column (11).

Gas Chromatography (GC)

GC is featuring the gaseous mobile phase in contrast to the liquid one in HPLC and suitable for the analysis of volatile and thermally stable compounds. Several detectors can be coupled with GC; for examples, flame ionization detector (FID), thermal conductivity detector (TCD), electron capture detector (ECD) and flame photometric detector (FPD). Similar to HPLC, it can be used for both qualitative and quantitative analyses. For example, the volatile chemical constituents of *Artemisia capillaris* were determined by gas chromatography-mass spectrometry and sub-window factor analysis. Seventy-five components were separated and 43 of them were qualitatively and quantitatively determined. Furthermore, *A. capillaris* was distinguished from *A. sacrorum* L., a possible substitute in traditional Chinese medicine by comparing the fingerprints with each other (12).

Through a pre-column derivation, GC can also be used for the analysis of non-volatile components. A GC method was developed for a simultaneous analysis of five major steroidal alkaloids, e.g., ebeiedine, ebeiedinone, verticine, verticinone and imperialine in *Fritillaria* species. Derivatization was carried out by trimethylsilylation of the hydroxyl-containing alkaloids. The developed assay was successfully applied to detect the major pharmacologically active alkaloids in three commonly used antitussive *Fritillaria* species: *F. cirrhosa*, *F. thunbergii* and *F. pallidiflora* (13).

Supercritical-fluid Chromatography (SFC)

SFC is characterized by the supercritical fluids used as the mobile phase to separate analytes with chromatographic columns. Supercritical fluids, e.g., carbon dioxide, can have solvating powers similar to organic solvents, but with higher diffusivities and lower viscosity (14). A major advantage of SFC is that it offers the advantage of liquid-like solubility, with the capability to use a non-

selective flame ionization detector (FID) or evaporative light scattering detector (ELSD). Analytes that cannot be vaporized for analysis by gas chromatography can be separated and detected using SFC.

Artemisinin and artemisinic acid were extracted from aerial parts of *Artemisia annua* by supercritical fluid extraction (SFE) with carbon dioxide modified with 3% (v/v) methanol, and analyzed by SFC-FID. Artemisinin and artemisinic acid were quantitatively extracted at a flow-rate of 2 mL/min in less than 20 min (15). The quantitative determination of both compounds was carried out by SFC-ELSD. This method allows mild extraction conditions and quantitative determination without further purification of the plant extract (16).

Capillary Electrophoresis (CE)

CE is used to separate mixtures of charged solutes species, for example, alkaloids, amino acids, peptides and proteins. Mixtures are separated in a narrow-bore fused quartz capillary tube under the influence of the electric field. It is used as an alternative or complementary technique to HPLC due to its high efficiency, speed of analysis, reduction in solvent and sample consumption and low operating cost (17). A capillary electrophoresis method was developed for the detection of five highly toxic alkaloids, e.g., strychnine, aconitine, brucine, mesaconitine and hypaconitine, in two commonly used herbal medicines. The buffer contained 40 mM ammonium acetate and 0.1% acetic acid in 80% methanol. Five alkaloids can be detected in 15 min by a single run (18). A nonaqueous capillary electrophoresis method was developed for the analysis of quinolizidine alkaloids in the roots of *Sophora flavescens*, *S. alopecuroides* and *S. tonkinensis*. A total of 10 alkaloids could be easily separated in 18 min. The running buffer composed of 50 mM ammonium acetate, 10% THF and 0.5% acetic acid in methanol (19). Micellar electrokinetic chromatography (MEKC), one of the most popular techniques in CE was used for the separation of 11 food dyes. The optimized electrolyte consists of 7.5 mmol/L TBS (pH 10.1), 10 mmol/L Brij and 15% acetonitrile. Complete separation of the 11 dyes was achieved in less than 9 min (20).

Spectroscopic Methods

The electromagnetic spectrum covers a wide range of wavelengths, frequencies and energies, and many analytical spectrometric techniques involve electromagnetic radiation. These techniques mainly include ultraviolet (UV)/visible spectrometry (wavelength 150-800 nm), infrared spectrometry (IR, wavelength 2-20 μ m; Near infrared 2-0.8 μ m), nuclear magnetic resonance spectroscopy (NMR, wavelength 1-100 m), power X-ray diffraction (PXRD, wavelength 2-0.1 nm) and mass spectrometry.

Ultraviolet/visible Spectrometry

UV spectrum is mainly used for the characterization of the chromophore of the analytes and quantitative determination of active components based on the absorbance. The total flavonoid contents of *Pueraria thomsonii* and *Pueraria lobata* from different collection areas were measured by UV method, which showed the average values of 1.93% and 7.8%, respectively (21). Besides the stand alone use, UV spectrum is often coupled with the chromatographic instruments, e.g., HPLC, as a commonly used detector providing online structure information (22).

Infrared spectrometry

IR is a useful indicator of functional groups in the molecules. IR may be used to generate fingerprints for the complex chemical composition in the herbs. The IR spectral data of *Angelica sinensis* from three origins were analyzed by a multivariate statistical method and the transformed data were visualized into the virtual chemical fingerprint plots by two-dimensional gray-scale images. The results showed that the same herb from different geographic origins can be efficiently differentiated (23). The Fourier Transform infrared (FT-IR) spectra of the American ginsengs of different geographical regions and the wild American ginseng have small differences, but their secondary derivative spectra provide obvious fingerprint features, especially in the range of 1750-400 cm^{-1} . The difference of the position and intensity of the bands can be used to identify and discriminate them from each other (24). FT-IR spectra and second derivative spectra were used to analyze the alcohol and sugar content of red wines produced by different manufacturers. For wines with less sugar, the intensity of absorption peaks assigned to C-O bond at 1120-1000 cm^{-1} becomes stronger when the alcohol content is higher (25).

Nuclear Magnetic Resonance Spectroscopy

NMR is based on the net absorption of energy in the radiofrequency region by the nuclei that have spin angular momentum and magnetic moment. Besides the well-known potency for structure determination, the NMR technique is also used for the evaluation and quality control of herbal extract. The NMR spectroscopic data of various extracts of St. John's wort (*Hypericum perforatum*) samples were found to be consistent with the corresponding IC_{50} values derived from the non-selective binding to opioid receptors, and multivariate data analysis of the proton NMR spectra was used to predict the pharmacological efficacy of St. John's wort extracts (26). NMR was also used for the quantitative

determination of active compounds in herbal medicines. For example, the ^1H -NMR spectrometry was used to analyze the terpene trilactone H-12 and the flavonol aglycon H-2' signals with methanol- d_4 -benzene- d_6 (65:35) selected as the optimal NMR solvent. The amount of terpene lactones and flavonol aglycons in various commercial Ginkgo products and leaves was determined. This method enabled the simultaneous analysis of terpene trilactones and flavonols and allowed simple and rapid quantification of these compounds in pharmaceutical Ginkgo preparations (27). Similar to UV detection, NMR may also couple with the separation instrument, such as LC/NMR.

Mass Spectrometry

Mass spectrometry sometimes is also regarded as a spectroscopic method. However, it is not based on the absorbance of any electromagnetic wavelength, but shows graphically as a plot of the number of ions of each mass detected. It has become an indispensable tool in the modern analytical laboratory. EI-MS was used for the authentication of *Gastrodia elata*, an herb used for the control of blood pressure. Based on the characteristic ion m/z 124 and its fragment ions 107 and 95 derived from the benzyl alcohol derivatives, the true species can be differentiated from the adulterants (28). Besides the stand alone usage, coupling MS with chromatographic techniques, such as GC and LC, has become increasingly popular.

Powder X-ray Diffraction

Spectrum of powder X-ray diffraction is generated through randomly-oriented microcrystals of solid material diffracting the x-ray. This method is used for confirmation of the identity of a solid material and determining crystallinity and phase purity. This method has been used for the comparison of chemical composition of pearls from sea water and freshwater and pearl stratum powder. The results showed that the main component of these pearl samples is calcium carbonate of aragonite type, but the latter contains small amount (less than 10%) of calcite type calcium carbonate (29). Powder X-ray diffraction can also be regarded as a fingerprinting method. Cortex Cinnamoni from ten sources was analyzed by this method. The result showed that these samples can be successful identified based on the topology and characteristic peaks in the spectrum (30).

Hyphenated Techniques

The rapid separation, qualitative and quantitative analyses of chemicals and simultaneous identification of the analytes have been an important issue in the

quality control of food and medicine. Thus combination of both chromatographic and spectroscopic methods has become increasingly popular. With a minute amount of herbal material and limited time, these hyphenated techniques allow an early recognition of known compounds and avoid the time consuming isolation process. Gas chromatography-mass spectrometry (GC-MS), Liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-nuclear magnetic resonance (LC-NMR) are three major hyphenated techniques applied in this field.

Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS is a combination of gas chromatography featuring the separation of volatile compounds by the carrier gas and mass spectrometry featuring the determination of structures based on the molecular ion and its fragmentation. *Angelica sinensis* is a commonly used herbal medicine and also a health food for women's care. GC-MS method was used to compare its chemical components with other two substitutes, e.g., *A. acutiloba* and *A. gigas*. Thirteen compounds were detected and significant variation of these compounds in three species was revealed (31). A rapid multiresidue method was developed for the analysis of insecticides, fungicides and acaricides in fruit juices. The residue levels were determined by gas chromatography with an electron-capture detector (GC-ECD), while the identity of these pesticides was confirmed by GC-MS (32). Headspace-solid phase microextraction (HS-SPME) is a useful method for sample pretreatment in GC analysis. This technique has been employed for sampling of volatile components and their volatile decomposition products occurring in herbal medicines. The identification and quantification were performed by GC-MS. As a fast and inexpensive technique for the isolation of organic analytes, HS-SPME with GC-MS can be successfully employed for the quality control of herbal medicines and other formulations containing herb extracts (33).

Liquid chromatography- Mass Spectrometry (LC-MS)

LC-MS is a hyphenated technique combining the advantages of both liquid chromatography and mass spectrometry. The ionization techniques used are mainly electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and thermospray ionization (TSI). Direct identification of peaks is possible based on the on-line UV and MS information in a chromatogram through the comparison with published data or reference compounds. A recent review has addressed the online electrospray and thermospray ionization techniques and their applications for the qualitative analyses of phenolic

compounds, saponins, alkaloids and other classes of natural products in botanical extracts (34). The application of LC-ESI-MS was investigated for the analysis of trans-resveratrol in red wine, grape skin, grape pomace, and winemaking byproducts. Chromatography was performed under isocratic reversed-phase conditions with the retention time less than 12 min. Qualitative analysis was performed in a negative mode. The results showed that the highest concentration was found in red wine, whereas wine made from grape pomace contained the lowest content (35).

Liquid Chromatography-Nuclear Magnetic Resonance (LC-NMR)

LC-NMR uses HPLC as the separation instrument while NMR serves as the detector. NMR data are of crucial importance for detailed structural investigation because of the comprehensive proton and carbon information and a variety of two-dimensional techniques (^1H - ^1H COSY, HMQC, HMBC, ROESY, etc.). Carbohydrates are major beer components, and their structural characterization by NMR alone is seriously hindered by strong spectroscopic overlap. LC-NMR was applied for the direct identification of carbohydrates in beer, which enables the rapid identification of dextrans up to 9 monomers. The significantly different oligosaccharide compounds in two beer samples reflect the different production conditions. Thus the use of hyphenated NMR may be the basis of a useful tool for the quality control of beer (36). An on-line coupling of high-performance liquid chromatography with nuclear magnetic resonance detection was used to analyze the root extract of the West African liana *Dioncophyllum thollonii*. The on-flow experiment allowed the identification of the naphthylisoquinoline alkaloid, dioncophylline A, while the second alkaloid, 5'-O-demethyldioncophylline A, was identified by subsequent application of stop-flow two-dimensional HPLC-NMR ROESY experiments (37).

Detailed comparison of the above chemical techniques is shown in Table 1.

DNA Technologies

Molecular authentication involves the use of DNA sequences or fingerprints to identify an individual. Theoretically, the genetic contents among individuals are not the same, even though their morphological characteristics are very similar. All health food and medicinal material except minerals can be authenticated by this approach. DNA-based markers are not affected by age and physiological conditions, and any part of the individual can be collected for analysis. Polymerase chain reaction (PCR)-based methods are currently dominating species identification and forensic investigation, as only a small amount of sample is needed. PCR-based methods can be classified into

Table 1. Common Chemical Techniques for Authentication and Quality Control

<i>Techniques</i>	<i>Principle</i>	<i>Application</i>	<i>Advantage</i>	<i>Limitation</i>	<i>Examples</i>
Thin layer chromatography (TLC)	Separation of analytes through a stationary phase coated plate by movement of a liquid mobile phase under normal pressure	Qualitative analysis of mixture by parallel comparison or quantitative analysis by density scanning	Low cost and easy operation	Low sensitivity, limited reproducibility	(7,8)
High performance liquid chromatography (HPLC)	Separation of analytes through a stationary phase filled column by movement of a liquid mobile phase under high pressure	Qualitative and quantitative analysis of mixture of nonvolatile compounds	Wide application to less volatile and ionic components and high sensitivity	Not useful for volatile and ionic components	(9,10,11)
Gas chromatography (GC)	Separation of analytes by movement of a gaseous mobile phase through a stationary phase capillary tube	Qualitative and quantitative analysis of mixture of volatile compounds	Wide application to volatile components and high sensitivity	Not useful for non-volatile components	(12,13)
Supercritical-fluid chromatography (SFC)	Supercritical fluids used as the mobile phase to separate analytes	Qualitative and quantitative analysis of mixture of low polarity compounds	Analytes that can not be vaporized for analysis by GC can be separated and detected using SFC	Not useful for highly polar components	(14,15,16)
Capillary electrophoresis (CE)	Separation of analytes through a narrow-bore fused quartz capillary tube applied with electric field	Qualitative and quantitative analysis of mixture of charged compounds	Fast and efficient separation of charged molecules and high sensitivity	Low sample loadability	(17,18,19,20)

Ultraviolet/visible spectrometry (UV)	Molecular absorption caused by electronic transition in the ultraviolet/visible region	Characterization of chromophores and quantitative determination of unsaturated organic compounds using Beer-Lambert Law	High precision and sensitivity for molecules bearing conjugated functional groups	Not useful for molecules bearing no conjugated groups and little information for whole structure	(21,22)
Infrared spectrometry (IR)	Molecular absorption caused by vibrational transition in the infrared region	Characterization of functional groups	High sensitivity for functional groups	Little information for whole structures	(23,24,25)
Nuclear magnetic resonance (NMR)	Nuclear absorption (change of spin states) in the radiofrequency region	Identification and structural analysis of organic compounds based on both proton and carbon signals	Comprehensive information on molecular structure	Medium sensitivity and high cost of the instrument	(26,27)
Mass spectrometry (MS)	Ionization and fragmentation of molecules including different ionization methods, e.g., EI, CI, ESI, MALDI	Identification and structural analysis of organic compounds based on the molecular ion and its fragmentation pattern	High sensitivity and efficient information on molecular weight and fragments	Limited potency for isomeric structures and high cost of the equipment	(28)
Power X-ray diffraction (PXR)	Randomly-oriented microcrystals of crystalline materials diffract X-rays and produce a pattern of rings on a distant screen	Confirmation of the identity of a solid material and determining crystallinity and phase purity	Sensitive for crystalline compounds	Limited for non-crystalline materials and high cost of the equipment	(29,30)

Continued on next page.

Table 1. Continued.

<i>Techniques</i>	<i>Principle</i>	<i>Application</i>	<i>Advantage</i>	<i>Limitation</i>	<i>Examples</i>
Gas chromatography-mass spectrometry (GC-MS)	Separation of the analytes by GC and using MS as the detector	Qualitative and quantitative analysis of mixture of volatile compounds and online structural elucidation based on MS	Efficient separation of volatile compounds and online structural information on molecular weight and fragments	Limited for non-volatile compounds, limited potency for isomeric structures and high cost of the equipment	(31,32,33)
Liquid chromatography-mass spectrometry (LC-MS)	Separation of the analytes by HPLC and using MS as the detector	Qualitative and quantitative analysis of mixture of nonvolatile compounds and online structural elucidation based on MS	Efficient separation of non-volatile compounds and online structural information on molecular weight and fragments	Limited for volatile compounds, limited potency for isomeric structures and high cost of the equipment	(34,35)
Liquid chromatography-nuclear magnetic resonance (LC-NMR)	Separation of the analytes by HPLC and using NMR as the detector	Qualitative and quantitative analysis of mixture of nonvolatile compounds and online structural elucidation based on NMR	Efficient separation of non-volatile compounds and comprehensive online information on structure	High cost of the equipment and medium sensitivity	(36,37)

fingerprinting and DNA sequencing. Fingerprint can be subdivided into whole genome fingerprinting and specific region fingerprinting.

Whole Genome Fingerprinting

This approach assesses the whole genome of an individual. Random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), amplified fragment length polymorphism (AFLP), and direct amplification of length polymorphism (DALP) are widely employed in species differentiation and phylogenetic studies. Whole genome fingerprinting is the simplest approach among DNA technologies. Moreover, it does not require any prior knowledge of DNA sequence of the sample. However, high quality of genomic DNA is required for generating reproducible results. TCM materials are usually dried or processed and DNA is somewhat degraded, making whole genome fingerprinting not always applicable.

Random Amplified Polymorphic DNA (RAPD)

A single arbitrary primer, generally ten nucleotides, is used to amplify genomic DNA under reduced stringent conditions. Amplification gives rise to DNA fragments that are generated when the primer randomly anneals on either strand. Due to the simplicity and convenience, it is extensively used in the analysis of food and medicinal material; for example, the RAPD markers that were generated with two primers could rapidly identify *Scutellaria galericulata*, *S. lateriflora* and *S. baicalensis* (38). Distinctive fingerprints of Dangshen (*Condonopsis pilosula*) of different localities in China were obtained by RAPD. Result showed that samples from the same province give similar fingerprints (39).

Direct Amplification of Length Polymorphism (DALP)

It is a modification of RAPD. DALP primers all share the same 5' core sequence for universal sequencing primer. It greatly facilitates the recovery and sequencing of polymorphic band (40). It was applied to authenticate *Panax ginseng* and *Panax quinquefolius*. A 636-bp DALP fragment was found in all *P. ginseng* but absent in all *P. quinquefolius* cultivars (41).

Simple Sequence Repeat (SSR)

Simple sequence repeat is tandem repeat of a few base pairs. SSR was first used to study cultivated species. It is similar to RAPD, but the primers are designed to flank microsatellite regions specifically and the annealing temperature is higher. The repetitive sequence can then be used as a probe in generating DNA fingerprints, such as for the differentiation of *P. ginseng* and *P. quinquefolius* (42). Another study found 61 useful SSRs for generating fingerprints of 30 potato cultivars from South America, North America and Europe (43).

Amplified Fragment Length Polymorphism (AFLP)

This technology is based on the amplification of a subset of restriction fragments of genomic DNA. Genomic DNA should be first digested with restriction enzymes. Resultant fragments are then ligated to synthetic adaptors and amplified with specified primers which are complementary to a selective sequence on the adaptor. The profile is determined by the number and composition of selective nucleotides. It was used to study the genetic diversity and phylogenetic characteristics of Japanese rice (*Oryza sativa* L.) cultivars that are used for brewing of Japanese rice wine. Result showed that the genetic diversity in sake-brewing rice cultivars was much smaller than that of cooking rice cultivars (44).

Specific Region Fingerprinting

Rather than assessing the whole genome, some investigators just use a particular region for species identification. Usually, the reproducibility of these tests is higher than that of whole genome fingerprinting.

Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP)

In this method, a defined DNA sequence is first amplified by PCR, and then selected restriction enzymes are used to digest the amplified region to generate fragments. The fingerprint depends on the absence or presence of restriction sites over a specific PCR product. With properly purified PCR product, the fingerprint is usually reproducible. Three orchid species, *Spiranthes hongkongensis*, *S. sinensis* and *S. spiralis*, were discriminated by their unique PCR-RFLP patterns. The introns located in NADH dehydrogenase genes of

orchid were PCR amplified. PCR products were cleaved with *HinfI* and analyzed by electrophoresis (45). In another study, ten fish species associated with U.K. food products were identified by the same approach. Specific profiles were generated by using *DdeI*, *NlaIII* and *HaeIII* restriction enzymes (46).

Sequence Characterized Amplified Region (SCAR)

A modified version of PCR-RFLP is sequence characterized amplified region (SCAR). SCAR may be revealed by sequencing the polymorphic bands from the whole genome fingerprinting. A pair of specific primers is used to amplify the polymorphic region under high stringent conditions. Identification of 5 *Panax* species (*P. ginseng*, *P. quinquefolius*, *P. japonicus*, *P. notoginseng* and *P. vietnamensis*) was achieved by five sets of species specific primers flanking 18S rRNA and chloroplast *trnK* (47). SCAR was also employed in detection of bacteria in food. Thirty different *B. cereus* strains, 8 other *Bacillus* strains and 16 other non-*Bacillus* strains were tested. It was showed that all non-*Bacillus* strains produced negative results (48).

DNA Sequencing

DNA sequencing is probably the most definitive means for species authentication. Protein encoding genes and gene spacers of nuclear, chloroplast and mitochondrial DNA are attractive targets for analysis. Internal transcribed spacer (ITS) of nuclear DNA and chloroplast genes are frequently used in plant identification and systematic investigation (49,50). Animal mitochondrial genomes have relatively rapid mutation rates (51). Several mitochondrial genes including cytochrome b, cytochrome oxidase I and 12S rRNA mitochondrial gene are targets for species specific sequences. These genes have conserved regions for making universal primers for amplifying the variable region in between. With vast amount of sequence data in public databases, it is possible to broadly classify the unknown sample even if the exact reference sequence is not available. 12S rRNA gene and cytochrome b fragments of five species of *Hippocampus* were PCR amplified. 12S rRNA sequence variation ranged from 0.79% to 6.61%, while cytochrome b sequence variation ranged from 1.98% to 19.47%. The variation is high enough for discriminating different *Hippocampus* species (52). Da-Huang (Radix et Rhizoma Rhei, medicinal rhubarb) has often been confused with the other *Rheum* species. By sequencing and comparing the *trnL-F* regions of chloroplast DNA of thirteen species of *Rheum*, a molecular marker of the medicinal species was found (53).

A list of the molecular techniques and their assessment is given in Table 2.

Table 2. PCR-based Methods for Authentication

Category	Technique	Principle	Advantages	Limitation	Example
Whole genome fingerprinting	Random amplified polymorphic DNA (RAPD)	Fingerprint is generated using a single arbitrary primer	Simple and fast	Influenced by degraded DNA, sensitive to experimental conditions	(38,39)
	Direct amplification length polymorphism (DALP)	Similar to RAPD but recovery of polymorphic DNA is simplified	Simple and fast	Influenced by degraded DNA	(41)
Amplified fragment length polymorphism (AFLP)	Short sequence repeat (SSR)	Fingerprint is generated using short sequence repeat specific primer	Simple and fast	Influenced by degraded DNA	(42,43)
		The restriction enzyme digested genomic DNA is ligated to specific adaptors and amplified by specified primers	Can detect a greater number of loci	Influenced by degraded DNA, procedure tedious	(44)

Specified region fingerprints	Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)	A defined region is amplified and digested by an appropriate restriction enzyme	High reproducibility and less affected by degraded DNA	Limited information provided	(45,46)
	Sequence characterized amplified region (SCAR)	A pair of specific primers is used to amplify the specific DNA under high stringent conditions	Simple and fast	Limited information provided	(47,48)
DNA sequencing	Sequencing	A defined region is amplified and sequenced	The most definitive identification method	May need to first clone the DNA	(52,53)

Conclusion

In this chapter, a brief introduction of chemical and molecular technologies is given. The choice of a certain technique depends on the nature of the material and the process of manufacturing the product. In general, molecular technology is highly effective for authenticating raw material and comparing two related materials. However, it may not be applicable for highly processed products or extracts, as DNA in them is degraded. Chemical technology may be used to assess the quality of the product, if one can correlate the fingerprints with the bioactive components. Thus, combined chemical and molecular technologies provide an accurate means for authentication and quality control.

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