

Natural Flavors and Fragrances

ACS SYMPOSIUM SERIES **908**

Natural Flavors and Fragrances

Chemistry, Analysis, and Production

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**Sponsored by the
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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.

ACS Books Department

Preface

Natural flavors and fragrances are an aspect of chemistry we enjoy every day. This fact is not lost on the flavor and fragrance industry and academic researchers around the world. Research into the production, sensation, and analysis of natural flavor and fragrance materials, as described in the chapters of this book, allows better development and application of natural aroma chemicals to the foods we eat and the fragrances we wear.

This American Chemical Society (ACS) Symposium Series volume is based on a symposium entitled *Natural Flavors and Fragrance Ingredients: Chemistry, Analysis, and Production* that took place on September 7, 2003 at the 226th ACS National Meeting in New York City. At this same ACS National Meeting, the Division of Agricultural and Food Chemistry presented a flavor workshop and four other symposia related to flavor chemistry, demonstrating the strong desire for researchers to meet and discuss the state of the art in flavor and fragrance chemistry.

This volume begins with an overview chapter and then addresses aspects of flavor and fragrance production, isolation, analysis, authentication, composition, functionality, and application. The diversity of the chapters reflects the diversity of the chemistry that challenges flavor and fragrance researchers. The authors were also a diverse group from England, France, Germany, Israel, Japan, South Korea, and the United States.

We thank the symposium participants and the authors of these chapters for their efforts in bringing this volume to publication. We also thank the editorial and production staff of the ACS Books Department and the chapter reviewers who helped to make this volume a dependable resource for its readers.

Further thanks go to the corporate sponsors of this symposium (Givaudan Flavors, International Flavors and Fragrances, McCormick and Company, Inc., Quest International, and Symrise) whose generous support made the program a reality.

Coeditor Frey thanks Valerie Jacklin, Director of Ingredient and Flavor Technology at Pepsi-Cola Research and Development, for supporting the efforts required to organize the symposium and to prepare this volume.

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Natural Flavors and Fragrances

Chapter 1

Natural Flavors and Fragrances: Chemistry, Analysis, and Production

Carl Frey

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Valhalla, NY 10595

Despite extensive use of flavor and fragrances from artificial sources, natural sources continue to provide flavor and fragrance materials that are unique and effective. Natural flavor and fragrances sources vary from the home kitchen to plants that grow deep in the tropics. Production methods vary from traditional harvesting of wild crops to fermentation of natural substrates using the most modern biotechnological techniques. Research into the chemistry of natural flavors and fragrances furthers our understanding and appreciation of these materials that continually engage our senses of smell and taste.

Introduction

In the beginning, the only flavor and fragrance (F&F) materials available were those derived from natural sources. The light bouquet of rose petals, tingle of cinnamon bark, the fresh scent of a lemon. With the development of synthetic organic chemistry techniques in the early 19th century the world of synthetic F&F materials began and expanded but it has not totally replaced natural F&F. A growing segment of the public prefers natural foods to foods containing artificial additives and will pay a premium for natural foods (*1*). While modern synthetic methods permit the economical production of artificial F&F on a large

scale, the natural F&F industry remains important. Why is this so? How do the chemical, biological, and agricultural sciences support the natural F&F industry? What is the state of current natural F&F research? These are questions this chapter and the rest of this volume will attempt to address.

History

Natural F&F have their origins going back to the start of recorded time. The Egyptians used the tree resins frankincense (olibanum) and myrrh, burned as incense (the word perfume means literally, 'through smoke') to appease the gods (2). *The Classic Herbal*, written by Shen Nung in China about 2700 BC records the use of more than 100 spices. Ancient Greeks, Arabians, and Romans established an international trade in Chinese, Indian and Southeast Asian spices. Magellan left Spain with 5 ships and 260 men in 1519 and although he and most of his men did not live to see Spain again, the one ship that did survive brought back a wealth of cloves from present-day Indonesia that spurred global exploration for spices and other products by the Europeans (3,4).

Current Market

Today, the entire F&F industry constitutes a \$16 billion (US) market world wide. Even considering only the value of F&F derived from essential oils, natural F&F constitute approximately a \$2 billion annual business (5). The impact of F&F is best appreciated when you consider that a flavor or fragrance typically contributes only a very small part, both in cost and volume, to a finished product. Despite adding little cost, the fragrance of a soap or the flavor of a beverage is often the point of difference that directs a customer purchase. The typical cost contribution of natural flavor to a manufactured soft drink is on the order of \$0.01/liter.

Definition of Natural

In many respects the modern F&F industry has come a long way from its origins. It is no longer just about picking roses, peeling bark off of cinnamon trees or squeezing lemons. The modern F&F industry applies modern technology to all aspects of production and processing. With the application of technology comes the question: How much technology can we apply to a natural F&F before we transform it into an artificial one? Like many modern issues the answer is complex and it depends upon whom you ask. In the US, 'natural flavor' is defined by the Code of Federal Regulations, 21 CFR 101.22 as "the essential oil, oleoresin, essence, or extractive, protein hydrolysate, distillate, of any product of roasting, heating, or enzymolysis, which contains the flavoring

constituents derived from a spice, fruit juice, vegetable, or vegetable juice, edible yeast, herb, bud, bark, root, leaf, or similar plant material, meat seafood, poultry, eggs, dairy products or fermentation products thereof whose significant function in food is flavoring rather than nutrition". A subset of natural F&F materials are those which meet the criterion, 'Organic', defined in the US by the USDA National Organic Program (7 CFR 205), which further requires, in short, that the material originates from an organism having a genome unaltered by modern biotechnology, produced and processed without the use of synthetic pesticides, sewage sludge, synthetic fertilizers, or irradiation, and using farmlands in sustainable ways. Regulations differ slightly in Europe and other countries and are continually subject to review and change.

Regulatory Organizations

Users and producers of F&F materials span the globe. National and international governmental, industrial, academic, and professional organizations oversee the F&F industry regarding production, distribution, quality, regulation and safe use for F&F materials (Table I). Regulatory organizations categorize flavor chemicals as food additives and require minimum standards for safety, quality, purity and limits for impurities. The 1958 US Food Additives Amendment to the Federal Food, Drug, and Cosmetic Act established guidelines for food additive safety, requiring additives to be safe under the conditions of intended use. Substances that are 'generally recognized as safe' (GRAS) do not need additional FDA review if they are evaluated by chemical, medical and toxicological experts for safety. The Flavor and Extract Manufacturers Association of the US (FEMA) works with an expert panel of food safety experts to assess the GRAS status of new and existing flavor materials. The FEMA Expert Panel has recognized over 2000 flavor substances as GRAS (6). Meaningful regulation requires careful assessment of the exposure of the general population and sub-populations (children, elderly, ethnic groups) to specific F&F materials. Calculations of exposure are costly, labor intensive and typically provide conservative estimates of exposure, erring on the side of safety (7). Exposure calculations require reference data on the concentrations of aroma chemicals in raw and cooked foods. Aroma concentration data is reported in the technical literature and also compiled in the BACIS Volatile Compounds in Foods database (www.xs4all.nl/~bacis/). In order to put any safety risk from flavor materials in perspective, exposure calculations also need to compare the proportion of flavor material consumed by the public in traditional foods to the quantity used by flavor companies as an ingredient. The proportion is known as the 'consumption ratio'. Coffee, with an annual per capita consumption (in the US) of about 5 kg, naturally contains approximately 20 ppm of the roasted coffee note 2,6-dimethyl pyrazine. The average person therefore ingests about

Table I. Internet Links to Fragrance and Flavor Science and Industry

Agricultural and Food Division of the American Chemical Society (AGFD)	membership.acs.org/a/agfd/
American Society of Perfumers	www.perfumers.org
American Spice Trade Association (ASTA)	www.astaspice.org
Cosmetic Toiletries and Fragrance Association (CSTA)	www.ctfa.org
European Flavor and Fragrance Association (EFFA)	www.ffa.be
Food Chemicals Codex (FCC)	www.iom.edu/project.asp?id=4585
Flavor and Extract Manufacturers Association of the United States (FEMA)	www.femaflavor.org
Fragrance Materials Association (FMA)	www.fmafragrance.org
International Federation of Essential Oils and Aroma Trades (IFEAT)	www.ifeat.org
International Fragrance Association (IFRA)	www.ifraorg.org
Institute of Food Technologists (IFT)	www.ift.org
Joint Expert Committee on Food Additives (JECFA)	www.codexalimentarius.net/jecfa.stm
Monell Chemical Senses Institute	www.monell.org
National Association of Flavor and Food-Ingredient Systems, Inc. (NAFFS)	www.naffs.org
Research Institute for Fragrance Materials (RIFM)	www.rifm.org
Society of Cosmetic Chemists (SCC)	www.scconline.org/index.shtml
Society of Flavor Chemists (SFC)	www.flavorchemist.org
Sense of Smell Institute (SOSI)	www.senseofsmell.org
The Fragrance Foundation	www.fragrance.org
United States Department of Agriculture (USDA)	www.usda.gov

100mg of 2,6-dimethyl pyrazine each year from coffee alone and ingests more if they consume roast beef and beer, which also contain natural 2,6-dimethyl pyrazine. The total consumption by the entire US population of 2,6-dimethyl pyrazine from traditional foods therefore exceeds 25,000 kg/year (8). The total use of 2,6-dimethyl pyrazine, both natural and synthetic, added as an ingredient by US flavor companies is only 20 kg (9). The consumption of 2,6-dimethyl pyrazine from traditional foods therefore greatly exceeds the quantity added as an ingredient by a factor of 25,000/20 (i.e. the consumption ratio is 1,250). Reducing the content of 2,6-dimethyl pyrazine from manufactured flavors would do little to reduce its consumption by the general population.

Production

In comparison to artificial F&F chemicals, natural F&F materials typically cost more and are more prone to supply interruptions due to changes in weather, disease, politics and economics. The use of natural F&F materials persists as in some cases natural production is cost effective and in almost all cases the sensory complexity provided by natural materials is not easily duplicated using artificial materials. Natural citrus oils are for the most part produced as a by-product of juice manufacture. Juice manufacture is a business of enormous scale, with many tons of fruit passing through a single processing plant. The essential oil recovered from the juice process is obtained through highly efficient automated equipment that minimizes cost, resulting in oils costing only a few dollars (US) per pound. Production of vanilla from vanilla beans is at the other end of the production spectrum. The process is entirely manual. The vanilla orchid (*Vanilla planifolia*) is a vine that grows in tropical climates around the world. Although vanilla was first discovered in Mexico its cultivation there was limited. Attempts to grow vanilla orchids in Madagascar were initially limited in that although the vines grew well enough the plants produced no beans. The lack of beans was finally attributed to the lack of vanilla flower fertilization. The vanilla orchid has a flower of small dimensions. In Mexico, a native bee is perfectly suited to the pollination of the vanilla orchid flower. Madagascar, not having appropriate bees has turned to humans to pollinate the vanilla orchid. A human inserting a sliver of bamboo into the flower pollinates each orchid. After several months, the green beans are picked by hand, sorted by size and quality, blanched in hot water to deactivate naturally present enzymes that contribute to spoilage and then cured under controlled heat and moisture conditions to develop the aldehyde vanillin and many other compounds, both volatile and non-volatile that produce a complex vanilla flavor and aroma. When the magnitude of the many manual steps involved in Vanilla production is considered its resulting high cost per pound is best appreciated.

Sources

Natural F&F have their sources in a wide variety of plants, animals, fungi and microorganisms (Figure 1). All parts of plants are used. The bitter orange tree provides F&F material from the blossoms (neroli oil, containing linalool), the leaves and stems (petigrain oil bigarade, containing linalyl acetate), and the fruit (bitter orange oil, containing limonene). The white albedo of the grapefruit contains the bitter flavanoid naringin. Other plant sources include roots (ginger, containing pungent gingerol), rhizomes (iris contain alpha and gamma-irones), pollen covered stigma (crocus saffron containing safranal), seeds (anise seed containing anethole), buds (clove, containing eugenol), pits and nuts (bitter almond oil and peach kernel oil containing benzaldehyde), resins (olibanum containing alpha-thujene), and residues of yeast and fermented grapes (cognac oil containing ethyl octanoate).

The harvesting of animals for fragrance material is now illegal. In the past wild musk deer were killed for scent glands that yielded aromatic musk containing the cyclic ketone, muscone. The development of synthetic musks for the fragrance industry has helped save the musk deer from extinction. Ambergris, which has its origin in the intestinal tract of the endangered sperm whale is also no longer legal. It has a unique musky odor of extreme tenacity due in part to the tricyclic ether, ambrox, which is now available synthetically. Civet cat scent gland secretions are collected as civet paste, possessing a tenacious fecal odor, in part due to indole and skatole that become floral upon high dilution. Natural animal notes also simply serve as signals or warnings - the trimethyl amine of decaying fish or the crotyl mercaptan of a disturbed skunk are readily recognizable odors to all of us.

Lichens and mosses are extracted to isolate their aromatic components that are widely used in the fragrance industry. Oakmoss, the lichen *Evernia prunastri* (L.) grows on oak trees. Its extract has a penetrating sweet, smoky, earthy odor due to a complex mixture of many compounds, including the characterizing methyl 2,4-dihydroxy-3,6-dimethyl benzoate (evernyl), and 3-methoxy-2,5-dimethyl phenol (orcinol).

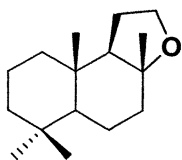
Biogenesis

Flavor and fragrance compounds can exist in nature their free forms, such as the terpenes of orange oil which are synthesized by the plant's metabolic processes. The terpenes can be released in a fine spray by merely squeezing the peel. Other F&F materials are only made available when enzymes, microbial reactions, or heat transform odorless substrates into aromatic materials. The grinding of mustard permits the mixing and reaction of odorless enzymes and

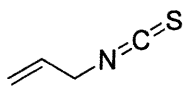
glucosinates (compounds containing both glucose and sulfur) present in the plant material to release tear prompting allyl isothiocyanate. Microorganisms produce enzymes, lipases, transforming fats in milk into cheese and generating aromatic methyl ketones, including 2-pentanone and carboxylic acids, including decanoic acid. The type of milk fat and microbes selected determines the flavor compounds produced that characterize and differentiate a Roquefort cheese from a Brie from a Cheddar. The non-enzymatic Maillard reactions between odorless sugars and amino acids found in meat can in the presence of heat (grilling, roasting) develop a variety of pyridines pyrroles, thiazoles, pyrazines and other aroma chemicals such as the meaty odorant 2-methyl 3-furanthiol. Meats and fish cooked in a variety of ways and then extracted and concentrated yield bouillon and extracts of high flavor impact. Understanding the Maillard reactions involved during traditional cooking has allowed chemists to create 'processed flavors'. Processed flavors start with pure sugars, amino acids and catalysts which are then heated, 'processed', into complex and concentrated meaty, brothy mixtures that can serve as the basis of meat flavors. Maillard reactions during the roasting of cocoa convert amino acids and sugars into pyrazines such as 2,3-dimethyl pyrazine and aldehydes such as 3-methylbutanal that have characteristic chocolate notes. An uncooked egg is almost odorless yet it contains all the raw materials for Maillard reactions. Upon heating, eggs generate, besides the characteristic hydrogen sulfide, over 100 volatile compounds: aldehydes, ketones, indoles, pyridines, pyrroles, and pyrazines. (10)

F&F compounds are usually not a significant part of plants. Typically they comprise <1%, sometimes much less than 1%, of the plant's total weight. They form from precursors that may constitute a large proportion of the plant. Maltol, a burnt sugar note, has carbohydrates as its precursor, vanillin has lignin as its precursor, beta-ionone, used in berry flavors, has carotenoids as its precursor, and the green notes, hexanal and (E)-2-hexenal, have the fatty acids, linoleic acid and linolenic acid, respectively, as their precursors.

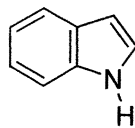
Flavor and fragrance chemicals are typically not related to the plant's primary internal processes for growing, photosynthesis or creating food stores. They are produced for secondary purposes such as protection or propagation. Bitter compounds like quinine in cinchona and caffeine in coffee protect the plant by discouraging animals from using them for forage. Aromatic compounds released by flowers attract pollinators to the plant, thus ensuring their continued survival. Prior to their release as flavor or fragrance compound many secondary metabolites reside in the plant chemically bound to glycosides. Treating plant material with enzymes designed to cleave the glycosidic bonds can release secondary metabolites that would otherwise not be available, increasing the yield of F&F materials recovered.



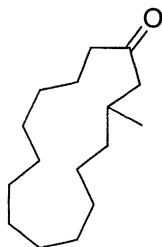
Ambrox
(ambergris)



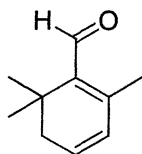
Allyl isothiocyanate
(mustard)



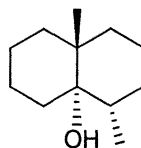
Indole
(civet & jasmine)



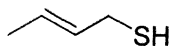
Muscone
(musk deer)



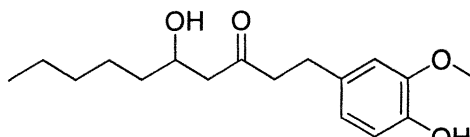
Safranal
(saffron)



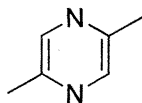
Geosmin
(algae)



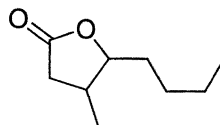
Crotyl mercaptan
(skunk)



6-Gingerol
(ginger)

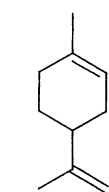


2,6-Dimethyl pyrazine
(roasted coffee)

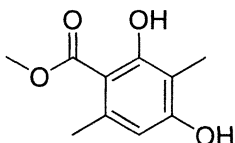


beta-Methyl gamma-octalactone
(oak)

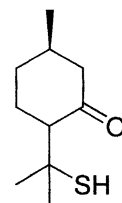
Figure 1. Sources of flavor and fragrance chemicals



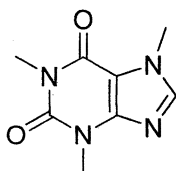
Limonene
(bergamot)



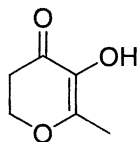
Evernyl
(oakmoss)



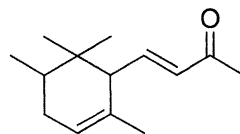
p-Mentha-8-thiol-3-one
(blackcurrant)



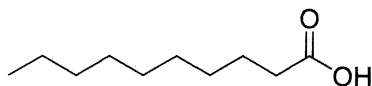
Caffeine
(cocoa)



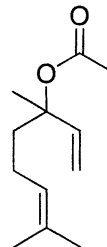
Maltol
(cooked sugar)



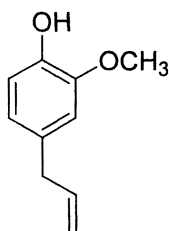
trans-alpha-Irone
(iris)



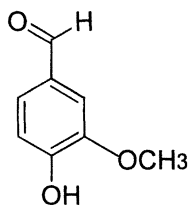
Decanoic acid
(cheese)



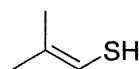
Linalyl acetate
(petitgrain)



Eugenol
(clove)



Vanillin
(vanilla)



3-Methyl but-2-en-1-thiol
(sun struck beer)

Figure 1 continued. Sources of flavor and fragrance chemicals

Biotechnology

The ability of microorganisms to produce traditional flavor compounds has been taken to the next step - using microorganisms to produce flavor compounds that either were difficult to isolate from natural sources or only available via synthetic organic chemical techniques. Using microorganisms and enzymes produced in large quantities with improved stability by biotechnology, today's biochemists can 'ferment' natural substrates to yield natural flavor compounds. Incubating natural cysteine and pulegone with the bacteria *Eubacterium limosum* can generate the potent blackcurrant odorant p-mentha-8-thiol-3-one (11). Biotechnology in the form of ELISA techniques can also provide sensitive methods for detecting F&F materials at extremely low levels. ELISA methods can detect <1 microgram/L of 2-methyl isoborneol in fish. The musty off-odor of 2-methyl isoborneol and geosmin, produced by fungi and blue-green algae, is a concern for the aquaculture industry (12).

Analysis objectives

F&F materials are subjected to a wide variety of analytical techniques for a wide variety of reasons. Understanding the composition of complex F&F materials and their wide variety of chemical structures leads to insights that permit synthesis of artificial F&F materials. The chemical basis of off-odors and off-tastes can help lead to processing and handling methods that minimize their production. Oxygen and light generate off-odors and off-tastes in foods due to autooxidation (hexanal generated by vegetable oil rancidity) and photooxidation (the skunky note in sun-struck beer, 3-methylbut-2-ene-1-thiol). Understanding the chemical basis of F&F material spoilage and degradation mechanisms can direct manufacturers to properly set product shelf lives and to control degradation mechanisms where possible. Monitoring the concentration of F&F materials in plants can direct growers to harvest only when plants contain the maximum amounts of volatiles. Analysis can determine the impact of packaging and its ability to protect or contaminate the foods and fragrances it contains. Plastic packaging materials, especially polyethylene can absorb (scalp) aromatics from the products they contain. Polyvinylidene chloride (Saran), polyester (PET), and oriented polypropylene scalp aromas to a lesser degree than polyethylene (13). Non-plastic containers can also affect product flavor. Aging wine in oak barrels permits oxygen from the air to enter the barrel and slowly oxidize (age) wine components. The wine also extracts compounds from the oak, such as whiskey lactone (beta-methyl gamma-octalactone) imparting a woody note. F&F materials derived from plants are prone to contamination from agricultural chemicals and processing aids. Orange oil recovered from juice manufacture can contain chlorinated terpenes, terpene chlorohydrins, resulting

from the use of excessively chlorinated water to wash the fruit during processing (14). Small differences in composition can serve as markers to determine the geographical origin of the same plant grown around the world. Compositional analysis can assess quality, purity and compliance with quality standards developed by regulatory groups. GCMS can authenticate the natural sourcing of F&F materials, differentiating them from inexpensive synthetic analogs by detecting minor impurities that only occur in synthetic F&F materials. The method can determine if sweet birch oil which naturally contains mostly methyl salicylate contains 1 to 5% or more methyl salicylate from synthetic sources (15).

Some F&F compounds have one or more asymmetric (chiral) centers. Natural materials with asymmetric centers usually are not racemic. The same compounds prepared from petrochemicals are racemic. Analyzing natural F&F chemicals for their enantiomeric composition using GC columns with optically active stationary phases, such as heptakis(2,3,6-tri-*O*-methyl)-beta-cyclodextrin, is a valuable natural authentication method. Chiral GC analysis often is further enhanced using a multi-dimensional GC that permits 'heart cutting' of a specific GC peaks for subsequent separation on a chiral GC column. Natural authentication by chiral GC can also differentiate the sources of the same chiral compound from different natural sources based on differing enantiomeric ratios. Limonene, which has a chiral center at the 4 position, has in bergamot oil a chiral ratio of 14(S):86(R) while in lemon oil it is 1(S):99(R), and in citronella oil it is 96(S):4(R). Some oils contain multiple chiral compounds with differing chiral ratios, increasing the opportunity for using chiral analysis to authenticate the oils as natural. Bergamot, lemon and citronella oils all also contain alpha-pinene that has a chiral center at the 1 position. In bergamot oil alpha-pinene has a chiral ratio of 72(S):28(R) while in lemon oil it is 62(S):38(R), and in citronella oil it is 23(S):77(R) (16).

Methods of Analysis

Sensory analysis is certainly the most fundamental way of assessing F&F materials. Panels of experienced tasters and odor evaluators direct the marketing strategies of all major food and fragrance manufacturers. Creative perfumers and flavorists develop their sensory skills over an apprenticeship of 7 to 10 or more years prior to qualifying for certification by flavorist and perfumer organizations. Many analytical tools available do not have a detector that is as sensitive to taste or odor as a human (or many animals for that matter). The challenge for researchers is to sift F&F information from all the data obtained from the various analytical techniques. One of the best ways to marry the best in analytical capability with the best in aroma detection is to combine the separation capability of a GC column with a human nose as a detector. The

resulting GC-olfactometry (GCO) can allow F&F researchers to quickly sort through many GC peaks to identify only those that have odor qualities of interest. Combining GCO with serial dilution techniques (i.e. Charm or AEDA, Aroma Extract Dilution Analysis) can determine the most odor active components in an extract (17). Components with a high Odor Activity Value (i.e. the ratio of the odorant concentration in the sample to the odorant's threshold in air) are the most important contributors to a sample's odor (or off-odor) (18).

GC, MS, NMR, IR, HPLC, TLC and other techniques, used both alone and in combination can analyze, authenticate and identify F&F materials. Table II lists the pros and cons of using various analytical techniques to authenticate F&F materials. GC using element specific detectors can detect sulfur or nitrogen compounds with high odor impact that occur at low concentration. It is not uncommon for F&F extracts of natural materials to contain hundreds of components. GC analysis of off-line 'rough cut' LC separations of a complex extract can help simplify the identification of the many individual components.

Stable Isotopic techniques

The isotopic distribution found in natural F&F compounds serves as a signature that analysts can use to differentiate natural compounds from synthetic analogs. The metabolic pathways that plants use to create carbon compounds discriminate between ^{13}C and ^{12}C . The $^{13}\text{C}/^{12}\text{C}$ ratio of the carbon dioxide the plant uses as a reactant differs from the $^{13}\text{C}/^{12}\text{C}$ ratio of the compounds the plant produces. Stable carbon isotope ratio analysis (SIRA) measures the $^{13}\text{C}/^{12}\text{C}$ ratio and can authenticate the natural or synthetic origins of some F&F materials. SIRA applied to isotopes of hydrogen, ^2H or D and ^1H can also serve to authenticate natural F&F materials. When combined with GC the on-line $^{13}\text{C}/^{12}\text{C}$ or D/ ^1H SIRA analysis can authenticate each component in a complex mixture such as a cinnamon bark oil (19).

Radioactive isotopic techniques

As living plants take in ambient carbon dioxide to create carbon compounds they incorporate the natural radioactive ^{14}C that is continually created in the atmosphere by irradiation of the atmosphere by cosmic rays from the sun. Once the plant is harvested or dies it no longer takes in new ^{14}C and the level of ^{14}C in the plant material begins to decrease at a rate equal to the half life of ^{14}C (5760 years). The small but measurable level of radioactivity from natural ^{14}C in F&F compounds isolated from plants is higher than the essentially zero level of ^{14}C in

Table II. Pros and Cons of Flavor and Fragrance Authentication Methods

method	pros	cons
GC sample: 0.1g or less high purity needed?: no test cost: ~\$100 equip. cost: ~\$30,000	Relatively fast and inexpensive. Customizable. Much published reference data. Can use element specific detectors (S, N).	ID of components requires another method, typically MS. Only for volatiles. Requires authentic references.
Chiral GC sample, purity requirements and costs similar to GC	Difficult to circumvent.	Requires authentic references. Columns cost more and are less durable than regular GC columns.
TLC sample: 0.1g or less high purity needed?: no test cost: ~\$25 equip. cost: ~\$200	Inexpensive and fast. Customizable. Non-destructive. Useful for non-volatiles.	Limited resolution capability. Need authentic references. High resolution methods cost much more.
HPLC sample, purity requirements and costs similar to GC	Fast. Non-destructive. Much published reference data. Useful for non-volatiles.	ID of components requires another method. Requires authentic references.
OR (optical rotation) sample: 1g or more high purity needed?: yes test cost: ~\$10 equip. cost: ~\$10,000	Fast. Much published reference data. As an HPLC detector costs more but lowers sample size requirements.	Only gives total OR, not a measure of enantiomeric ratios. Only for chiral materials.
Carbon SIRA sample: 0.1g or less high purity needed?: yes test cost: ~\$100 equip. cost: ~\$200,000	Can authenticate some compounds (vanillin). Can be combined with GC for peak-by-peak analysis.	Does not have application to many F&F materials. Equipment requires careful calibration.
Hydrogen SIRA sample, purity requirements and costs similar to Carbon SIRA	More application than Carbon SIRA. Can be combined with GC for peak-by-peak analysis.	Deuterium F&F analogs are available to adjust the overall ratio of synthetics to pass test.
Carbon-14 sample: 1 to 3 g high purity needed?: yes test cost: ~\$200 equip. cost: ~\$200,000	Provides definitive results unless sample is diluted with radioactive components. No database needed.	F&F materials synthesized from natural materials (i.e. citral from turpentine) will pass test as 100% natural.
D-NMR (SNIF-NMR) sample: 0.5 to 2 g high purity needed?: yes test cost: ~\$1000 - \$1500 equip. cost: ~\$500,000	Reliable when used with a reference database. Difficult to circumvent.	Expensive. Requires database. Natural F&F from new sources (i.e. not in reference database) may test as non-natural.

F&F compounds prepared from petroleum. The carbon in petroleum is millions of years and many half-lives older than the carbon in growing plants and is no longer radioactive.

Isolation and Interaction Effects

F&F compounds are sometimes available at high purity (such as orange oil expressed from orange peels) and at other times are only available at low concentration in a substrate (such as the flavor of bread crust which is only generated upon baking). Since most flavors and fragrances are active at extremely low levels, most F&F analyses begin with a step to concentrate the F&F to more readily detectable concentrations. The selection of concentration techniques needs to consider that many F&F compounds are labile and prone to reaction and degradation when exposed to heat or changes in pH. Distillation at low pH can hydrolyze esters. Enzymes naturally present in plant material can react with F&F that are also naturally present. If the desire is to isolate the natural the aroma of a plant it is important that the enzymes native to the plant that could degrade the natural aroma are either not activated (by collecting the headspace of an intact plant, for example) or inactivated by mild heat or methanol or concentrated salt solutions. Techniques such as liquid CO₂ extraction, SPME, purge and trap, low temperature vacuum distillation, and solvent extraction can isolate F&F compounds without generating too many reaction products (20). When using CO₂ recovered from fermentation, liquid CO₂ extraction has the added benefit that the solvent contains no artificial residues typically associated with organic solvents, leaving the resulting extract natural and organic (21).

Isolation also has to consider the interactions of substrates with the flavors and fragrances they contain. Collecting natural odors from human skin or breath is complicated by the low concentrations of odorants to be detected and the simultaneous emission of relatively high concentrations of water vapor. Skin emanations can be collected by washing the skin with ethanol or by rubbing the skin with glass beads which are subsequently thermally desorbed (22). Fats, carbohydrates, water, and proteins in foods all influence the stability, release and sensory perception of flavors they contain. A flavor made for an ice cream will taste different if put into hard candy. The partition coefficients of aroma molecules typically will favor an oil phase, restricting the amount of aroma molecules released to the air and eventually to the odor receptors in the nose. Carbohydrates also affect the partition coefficients of aroma molecules but the effect is variable. Carbohydrates bind some flavors and release others into the air, similar to a salting-out effect. Proteins, being macromolecules provide many sites to bind aroma molecules by hydrogen bonding or van der Waals forces. The amine groups of proteins are also available to react with aldehydes (23).

Aromatherapy

The use of aromas to stimulate healing and peace of mind is known as aromatherapy. Much research has focused on linking and quantitating the 'feel good' (or bad) effect of aromas to physiological changes within the human body. Chemical constituents of inhaled vapors of natural essential oils can be detected in plasma and cell membranes of aromatherapy participants and can induce measurable changes in EEG activity. The effects are more than psychosomatic as even those afflicted with anosmia demonstrate changes in EEG activity (24).

Odor and taste reception

The most basic understanding of F&F materials has to consider how the human senses of taste and smell detect and differentiate chemical compounds. Flavor is a complex response to a combination of receptors: salt, sweet, bitter, sour, and umami (Japanese for 'tastes good'), odor, and trigeminal (heat, cooling, pain, temperature, tingling, astringency, and pungency) (25). Texture, viscosity, mouth-feel and even the audible crunch of crisp foods and the fizz of carbonated beverages contribute to the sensory experience. Human taste sensory cells for bitter, sweet and umami compounds involve G-protein coupled receptors and salty and sour compounds are detected via effects on ion channels of taste receptor cells. Establishing Structure Activity Relationships (SAR), which determine the chemical structure needed to produce a desired sensory effect, can lead researchers to design molecules of specific sensory character from 'scratch' or to design compounds that will block the sensation of specific tastes. SAR studies led to the development of a better understanding of the structures that produce the pungency of Asian peppers. A newly synthesized molecule, isobutyl undeca-2E, 4E, 8Z-triene amide, designed based on SAR learnings, was found to be pungent, as predicted (26).

Non-Volatiles

While by definition, aroma compounds need to be volatile enough to be inhaled into the nose, flavor compounds can either be volatile or non-volatile. Volatile compounds typically have molecular weights below 300 Daltons. Non-volatile flavor compounds can have molecular weights well over 300. Detection of non-volatiles requires the use of TLC or HPLC either alone or in tandem with an identification technique. HPLC-NMR can identify non-volatile coumarins, sterols, fatty acids and psoralens, in citrus oils, such as the substituted psoralen byakangelicol, found in lemon peel oil. Non-volatiles can act as odor fixatives, impacting the release of a perfume from the skin. Non-volatiles also serve as authentication markers that are difficult to adjust or circumvent (27).

Summary

The intent of this chapter was to give the reader and appreciation for the many facets of the chemistry, analysis and production of natural flavors and fragrances. It is a field where each one of us who can smell and taste are active participants. New foods, plants, processes, packages and aromas are developed each day. Keeping researchers abreast of the many new facets of the natural F&F field have led to development of this chapter and the ACS symposium that served as the basis of this book.

References

1. Sloan, A.E., *Food Technology* **2003**, 57(4), 30-49
2. Gibbons, B., Psihoyos, L., *National Geographic*, **1986**, September, 324-362
3. Dziezak, J.D., *Food Technology* **1989**, 43(1), 102
4. Bergreen, L. *Over the Edge of the World*, Willam Morrow, New York, NY, 2003
5. Short, P. *Chem. Eng. News* **2002**, 80(18), 32-37
6. Smith, R.L., Doull, J., Feron, V.J., Goodman, J.I., Munro, I.C., Newberne, P.S., Portoghese, P.S., Waddell, W.J., Wagner, B.M., Adams, T.B., McGowen, M.M. *Food Technology* **2001**, 55(12), 34-55
7. Hall, R.L., Ford, R.A. *Food Additives and Contaminants* **1999**, 16(11), 481-495
8. Stofberg, J. *Perfumer and Flavorist* **1983**, 8, 53-62
9. Lucas, C.D., Putnam, J.M., Hallagan, J.B., *1995 Poundage and Technical Effects Update Survey*, Flavor and Extract Manufacturers' Association of the United States, Washington, DC 1999, p 90
10. Umamo, K., Hagi, Y., Shoji, A., Shibamoto, T., *J. Ag. Food Chem.* **1990**, 38, 461-464.
11. Kerkenaar, A., Schmedding, D.J.M., Berg, J., US Patent 5,182,194, 1988
12. Phlak, L.C., Park, E.S. *J. Agric. Food Chem.* **2003**, 51, 3731-3736
13. Brody, A.L. *Food Technology* **2002**, 56(6), 124-125
14. Weiss, E.R.; Braddock, R.J.; Goodrich, R.M.; Gregory, J.F. III; Pika, J., *J. Food Sci.* **2003**, 68(6), 2146-2149
15. Frey, C. in *Flavors and Fragrances: A World Perspective.; Proceedings of the 10th International Congress of Essential Oils, Fragrances, and Flavors*; Lawrence, B.M; Mookherjee, B.D; Willis, B.J. Ed.; Elsevier Science Publishers, Amsterdam, pp 517-524
16. Mosandl, A., Hener, U., Kreis, P., Schmarr, H.G. *Flav. Frag. J.*, **1990**, 5, 193-199

17. Acree, T.E., *Analytical Chemistry* **1997**, March 1, 170A-175A
18. Grosch, W., *Flav Frag. J.* **1994**, 9, 147-158
19. Swenig, S; Hener, U.; Mosandl, A., *Eur. Food Res. Tech.* **2003**, 217(5), 444-448
20. *Flavor Analysis: Developments in Isolation and Characterization*; Editors, Mussinan, C.J., Morello, M.J. ACS Symposium Series 705; American Chemical Society, Washington, DC, 1998, pp 1-123
21. Moyler, D.A. *Flav. Frag. J.*, **1993**, 8, 235-247
22. Bernier, U.R., Booth, M.M., Yost, R.A. *Analytical Chemistry* **1999**, 71(1), 1-7
23. Leland, Jane V., *Food Technology* **1997**, 51(1), 75-80
24. Buchbauer, G. in *Proceedings of the 12th International Congress of Flavours, Fragrances, and Essential Oils*, Woidich, H., Buchbauer, G. , Ed. Austrian Association of Flavour Fragrance Industry. Vienna, pp 34-43
25. Prescott, J., Stevenson, R.J., *Food Rev. Int.* **1995**, 11(4), 665-698
26. Galopin, C.C., Furrer, S.M., Goeke, A., in *Challenges in Taste Chemistry and Biology*, Hofmann, T., Ho, C-T., Pickenhagen, W. Eds., ACS, Washington, DC, 2003,pp 139-152
27. Sommer, H., Bertram, H.J.; Krammer, G., Kindel, G., Kuhnle, T., Reinders, G, Reiss, I., Schmidt, C.O., Schreiber, K., Stumpe, W., Werkhoff, P., *Perfumer and Flavorist* **2003**, 28(1), 18-34

Chapter 2

Production of Natural Flavor and Fragrance Ingredients in 2003: Massoia, Orris, and Vetiver Oils

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Most natural flavor and fragrance ingredient developments have been made:

- Over the past 100 years, with the progressive modernization of essential oil distillation and extraction technologies.
- Over the past 50 years, taking advantage of the spectacular development of analytical tools, a comprehensive knowledge of natural products compositions has been accumulated

Emphasis is now put on sourcing the raw materials. The understanding of the market in high value flavor and fragrance raw materials is becoming very important. This study focuses on 3 raw materials, Massoia, Orris and Vetiver. Cultivation, post-harvesting practices and chemical characteristics of each particular raw material are discussed.

A modern company manufacturing natural products for perfumes and flavors needs to accomplish the integration of all steps of production including the mastery of the raw material source. The time is over that a manufacturer was just producing an essential oil after purchasing a botanical species from a broker.

Several examples of this approach are detailed below. These represent products for which teamwork has been initiated between the manufacturer's sourcing department and its research and development department on the one hand and the raw material supplier on the other hand.

- Massoia bark essential oil, raw material to make Massoia lactone, which suffered from considerable quality variations. Sourcing a reliable supplier required numerous analyses and laboratory distillations of different barks.
- Orris rhizomes, to manufacture orris concrete (butter) and absolute. The optimization of the ageing process has been effected after having analyzed and processed rhizomes at all stages of growing and preparation.
- Vetiver essential oil, to manufacture derivatives like vetiveryl acetate. Manufacturers prefer oil with a high alcohol content. Finding an alternative to vetiver Haitian essential oil, rich in vetiverol, required analyses and a new manufacturing process.

Massoia Essential Oil's Controversial History

Massoia lactone is a very interesting natural isolate obtained from massoia essential oil. It brings a very strong coconut-peach-like aroma. For a long time, there was a great deal of misunderstanding with regards to the botanical origin of massoia essential oil.

Two barks, Massoia and Lawang, were historically distilled in New Guinea. Many authors and traders were confused about true nature of massoia oil as it was frequently confused with Lawang oil (1,2). The controversy stopped after the works of Abe (3) were published in 1937, of Meijer in 1940 (4) and of Salverda in 1937-1938. The last author completed an expedition to New Guinea and collected authentic specimens of true massoia. The two essential oils were produced. Lawang, coming from various *Cinnamomum* species. It is rich in eugenol, and methyleugenol. Massoia contains no eugenol and comes from *Cryptocarya massoia* (fam. Lauraceae)

In 1940, Meijer (4) isolated Massoia lactone, 5,6-Dihydro-6-pentyl (2H)pyran-2-one (Dec-2-en-5-olide), C₁₀H₁₆O₂, from true massoia oil. In 1954, Abe and Sato (5) confirmed the structure. In 1968, Cavill & al. (6) isolated a

second lactone from massoia bark, the homologous C₁₂H₂₀O₂ in 1983. Garnero & Joulain (7) examined the essential oil of massoia in detail. In 1990, Bernreuther & al. (8) published an enantioselective analysis of massoia lactone. They identified the naturally occurring massoia lactone as R(-)-dec-2-en-5-olide.

Purchasing Massoia Essential Oil

Purchasing massoia essential oil to manufacture massoia lactone, presents the problem of sorting through a wide spectrum of qualities. The objective of this study was to obtain oil with a high concentration of massoia lactone with the authentic absolute configuration. A large number of samples were examined coming from Indonesian distillers as well as from distributors. The massoia lactone content ranged from 49% to 74%. The C₁₂ lactone homolog content ranged from 12% to 4100. The enantiomeric ratio of massoia lactone ranged from an R(-)-massoia lactone content of 75% up to an R(-)- massoia lactone content of 98%. No consistency in essential oil content or composition was found at this stage.

The massoia tree occurs in a few varieties, distinguished by those with thin bark and those with thick bark. Working with selected producers who provided oils of the right composition, i.e. a high percentage of massoia lactone along with the right enantiomeric ratio, yielded different types of barks that were subjected to lab scale extraction and analysis in the laboratory.

Massoia Bark Gathering and Processing

Massoia trees are mainly grown in Irian Jaya / Papua provinces, in villages including Jaya-Pura, Merauke, and Fak-Fak. Massoia trees are grown throughout the province. Quantities available permit a production of at least 5 tons of oil per year. The plants are grown, at least for now as a sustainable crop. Bark cutting is done the whole yearlong. Fresh bark is gathered and checked in villages by collectors. The quality control step at this stage consists of looking at the bark and smelling it. The collected fresh barks (Figure 1) are dried under the sun before shipping to distilleries. The field distillers report that no research is done on massoia bark by any local or state agricultural laboratory. Analytical differences in the levels of the C₁₀ lactone, C₁₂ lactone, and benzyl benzoate as well as the chirality of C₁₀ lactone and C₁₂ lactones are mainly linked with origins/locations where the trees are grown.

Initial Work to Differentiate Massoia Bark Oils by Origin

Massoia essential oils prepared from barks of various origins were analyzed after laboratory extraction. Table I summarizes the compositional analysis

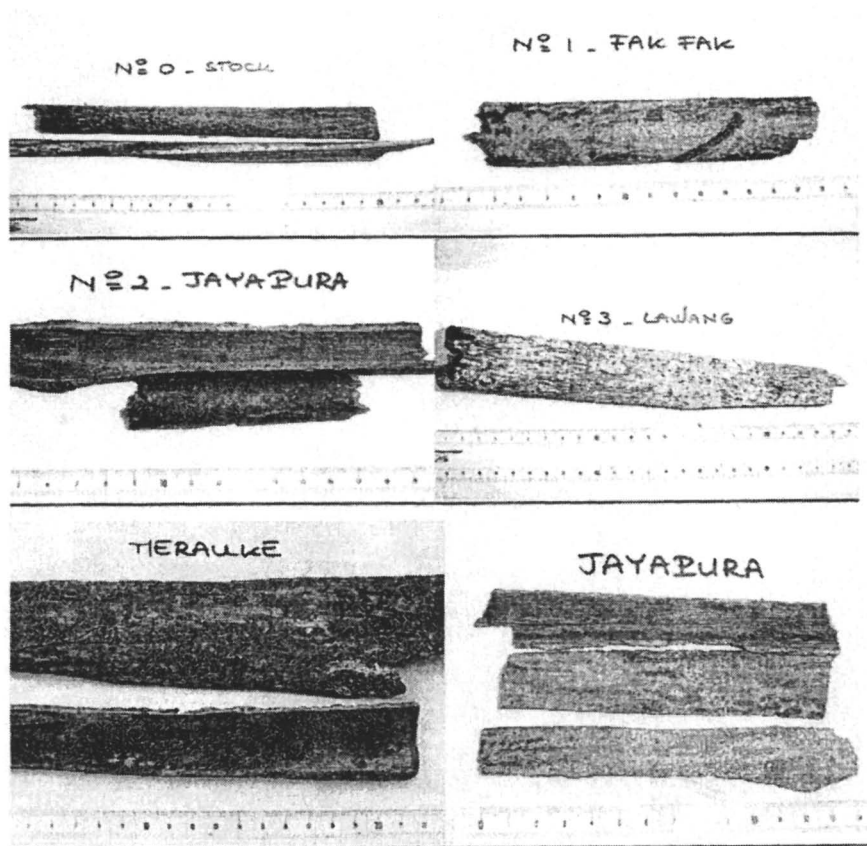


Figure 1. Initial samples of Massoia barks of different origins:

Table I. Compositional Analysis of Massoia Oils, First Sampling

Origin of barks	C10 lactone content %	e.e.%* R(-)C10 lactone	C12 lactone content %	e.e.% R(-)C12 lactone
European broker	65.9	97.7	7.0	73.6
Fak-Fak	55.2	82.8	35.9	49.0
Jaya-Pura	29.5	93.8	59.7	89.8
	Eugenol %		Methyl eugenol %	
Lawang (so-called massoia in the past)	23.5		66.2	

$$*e.e.\% = \text{enantiomeric excess} = \frac{\text{major} - \text{minor}}{\text{major} + \text{minor}} \times 100 = \frac{R(-)ML - S(+)ML}{R(-)ML + S(+)ML} \times 100$$

$(R-)ML = R(-)\text{-massoia lactone}$

$(S+)ML = S(+)\text{-massoia lactone}$

results for massoia lactone, the C12 homolog (and their stereochemistry) for barks of varying origin.

From the data in Table I, we confirm that the Lawang bark essential oil is really different from massoia bark oil, containing mostly a mixture of eugenol and methyleugenol and no massoia lactone. These first analyses made after distillation of actual barks, also confirmed that the real massoia essential oils are very different from one collection town to another. Oil obtained from bark purchased from a broker in Europe is different again. The variations occur in both the massoia lactone content and in its stereochemistry. No quality consistency was found with these purchased barks or their essential oils.

Second Attempt to Source Authentic Massoia Oil

Continuing the sourcing search finally a yielded a consistent supplier of authentic massoia bark oil. This supplier provided two qualities of barks, again

Table II. Compositional Analysis of Massoia Oils, Second Sampling

Origin of barks	C10 lactone content %	e.e.% C10 lactone	C12 lactone content %	e.e.% C12 lactone
Merauke	81.2	95.8 R(-) massoia lactone	8.6	71.6 R(-)C12 lactone
Jaya-Pura	79.5	88.4 S(+) massoia lactone	9.7	91.2 S(+)C12 lactone

under the names of their collection towns. The results of the extraction of these barks (Table II) confirmed the distiller's contention that the quality of the oil obtained is predictable based on the type of bark used to prepare the oil.

These barks were prepared in 2 ways: by extraction without the use of heat, and by water-distillation, the typical Indonesian process. The resulting oils are similar to one another. The hydrodistillation process does not modify the oil in any way.

The oil prepared from bark obtained from Jaya-Pura origin during this second sourcing attempt is very different from that obtained previously. Its chirality is also different, rich in S(+) massoia lactone. Investigation of this oil continues.

Massoia Bark Sourcing Conclusions

An intensive massoia sourcing and R&D program overcame raw material inconsistency problems encountered previously. The improved knowledge of this item combined with the identification of a reliable source has permitted an increase in the sales of massoia bark oil.

Orris Rhizomes in Perfumes and Flavors

Orris derivatives are used in expensive perfumes for their delicate, violet-like, woody aromas. In flavors, orris derivatives are mainly used to modify fruity flavors. In both types of applications, orris derivatives are formulated at extremely low levels, where they still bring warmth and diffusiveness. Their price is very high. However, as far as they are formulated in extreme dilution, they are still cost effective and in strong demand by perfumers and flavorists. The orris products family is characterized by a rather simple process flow chart (Figure 2). Two sources of orris raw materials are processed:

- Italian orris rhizomes (*Iris pallida* L.): most are dried and aged unpeeled.
- Moroccan orris rhizomes (*Iris germanica* L.): most are peeled before drying and ageing.

Orris rhizomes of both Italian and Moroccan origins are hydrodistilled in water (9) to provide an essential oil. Orris essential oils, which are solid at room temperature, are frequently misnamed 'concretes'. They also go by the name of 'orris butter', which is explained by their butter-like texture. A subsequent purification step by alcohol extraction into an absolute eliminates the fatty acids from the essential oil (butter, concrete).

The classical cultivation of orris rhizomes and their subsequent preparation into an essential oil, is a very long process, consisting of :

- 3 years for the plant to grow in the fields
- followed by another 3-year period during which the valuable irones develop up to a satisfactory level. See Figure 2.

Improved Development of Irones in Orris Rhizomes

Irone formation in ageing rhizomes has been described by Jaenicke & al. in the early 1980's. (10,11) The objective of this study was to obtain a better understanding of the ageing mechanisms in orris and the development of an improved industrial pathway that could generate a maximum of the desirable irones. Accomplishing this objective required developing analytical methods to quantify the irones in lots of orris rhizomes and to also quantify potential irones present in the rhizomes as precursors.

Method for Quantifying Irones in Orris Rhizomes

Ground orris rhizomes to which an internal standard was added were extracted with hexane under reflux (Figure 3). After hexane concentration, irones were quantified by gas chromatography, following the French test standard NFT 75-424 (October 1996). This lab method provides results that correlate well with data obtained for actual industrial scale productions. The laboratory quantification method is useful for predicting industrial yields and is used as a guide for purchasing lots of orris rhizomes.

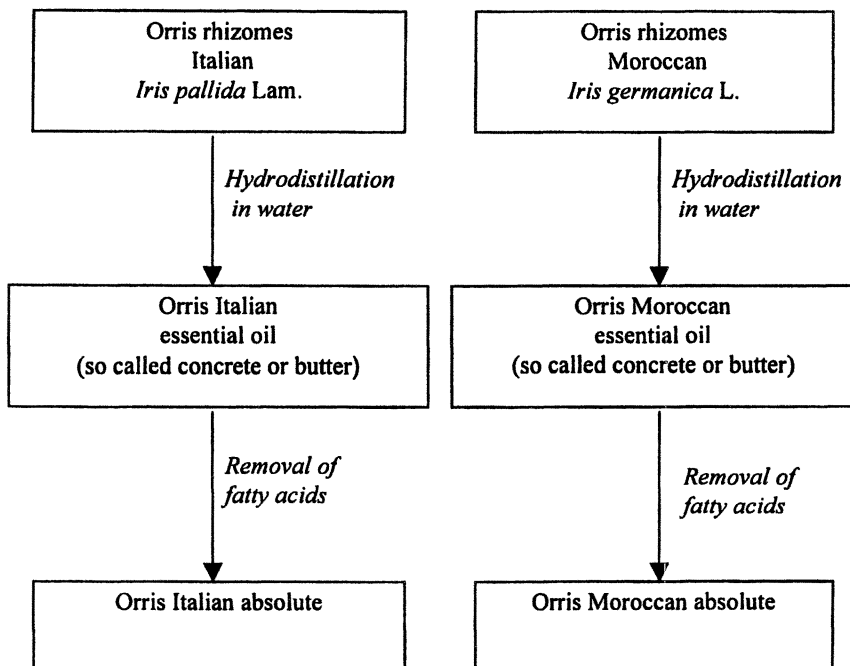


Figure 2. Orris Derivatives ProcessFlowChart

Method to Quantify Irones Precursors in Orris Rhizomes

The adopted HPLC quantification method is derived from the work of Bicchi & al. (12). It includes the purification of an irone precursors fraction (Figure 5), obtained by extraction of freshly gathered orris rhizomes (Figure 4). The level of irone precursors determines the potential of irones still not developed in the ageing orris rhizomes. The third chromatogram demonstrates that even after a long ageing period, irone precursors are not completely transformed to irones (Figure 6).

HPLC Experimental Conditions for Orris Rhizome Analysis

HPLC was carried out on a Waters 600 system provided with a UV detector. A Spherosil LC18 5U reverse phase column (25 cm x 4.6 mm i.d., 5 µm; Supelco, Bellefonte, PA, USA) was used. A linear gradient of methanol: water from 3:7 to 8:2 in 11 min, followed by 15 min of isocratic elution and then by a further gradient from methanol: water (8:2) to pure methanol in 5 min was applied. The eluent flow-rate was 1 mL/min, the injection loop volume was 20 µL and the detection wavelength was set at 254 nm.

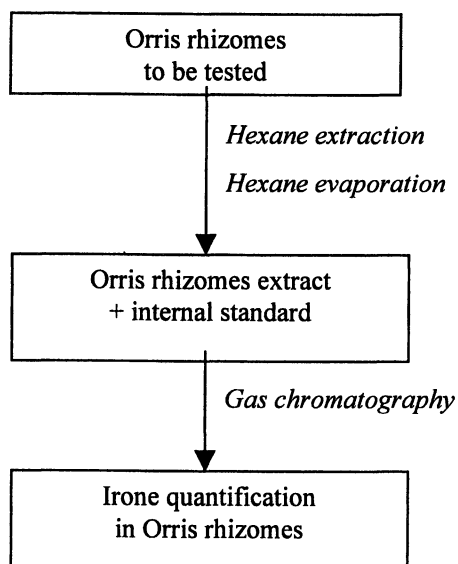


Figure 3. Analysis Scheme for Irones in Orris Extracts

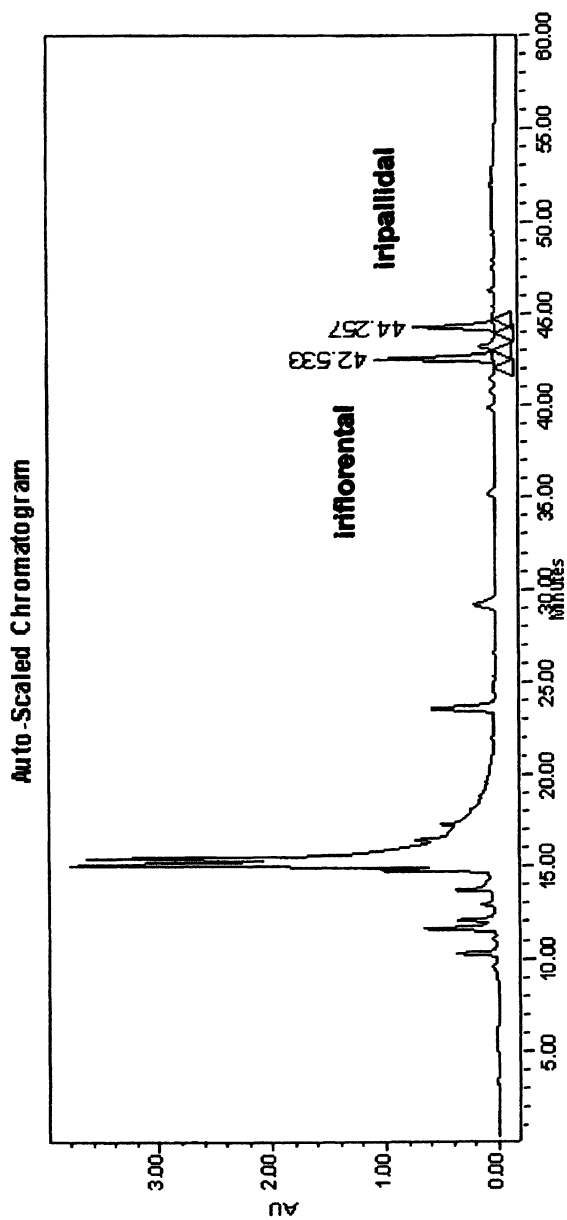


Figure 4. HPLC of an Extract of Freshly Gathered Orris Rhizomes

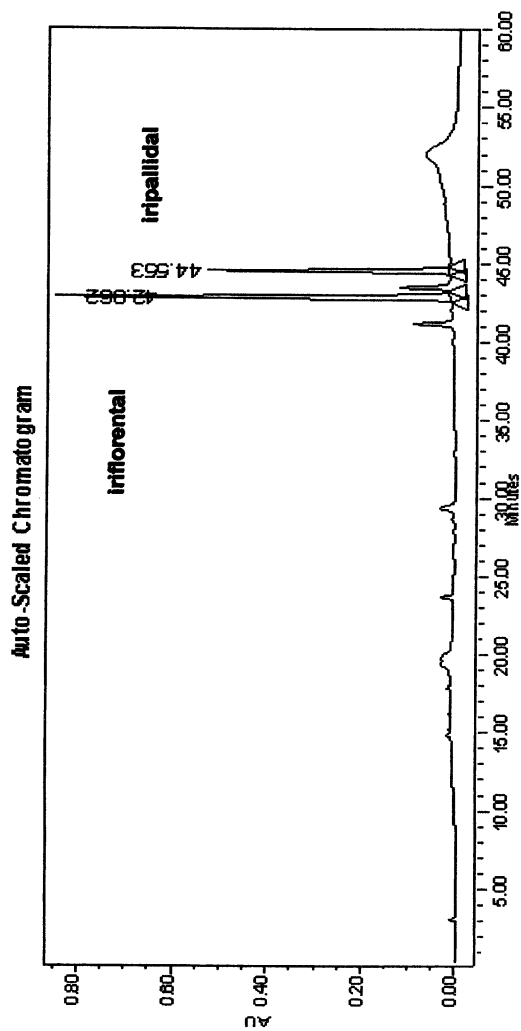


Figure 5. HPLC of Purified Irone Precursor Fraction.

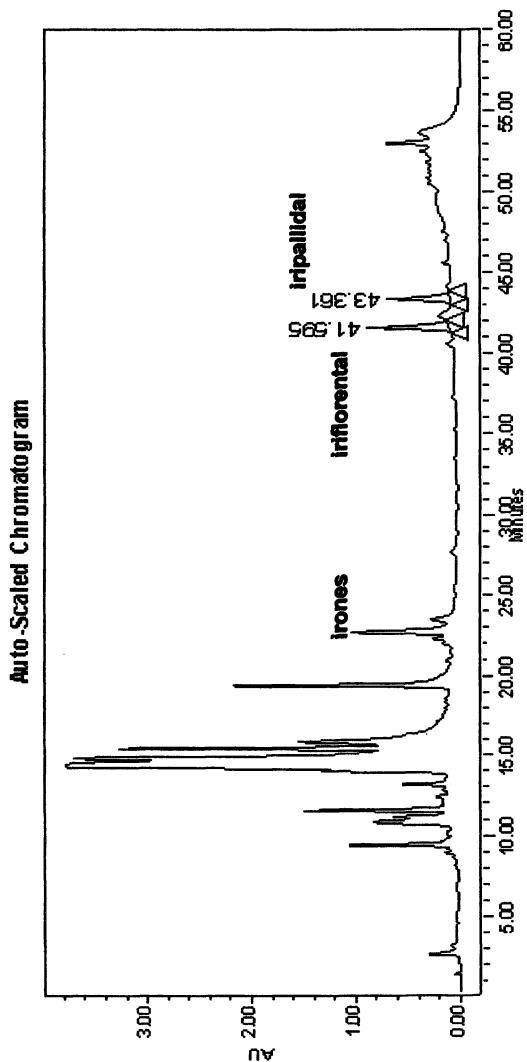


Figure 6. HPLC of an Extract Orris Rhizomes Aged 4 Years. Note that even after a long ageing period, irone precursors are not completely transformed into irones.

Traditional Ageing of Orris Rhizomes and its Effect on Iron Formation

A large number of orris rhizomes were analyzed from 2 Italian sources:

- from undried freshly harvested rhizomes grown one to three years in the field.
- to conventionally dried rhizomes aged one to four years.

For each lot of orris rhizomes, both the existing irones and the irones potentially present as a precursors have been quantified. The results obtained for dried Italian orris rhizomes aged from one to four years are listed in Table III.

Table III. Analysis of Italian Orris Rhizomes

	1 year of aging	2 years of aging	3 years of aging	4 years of aging
Iron content (ppm)	500	600	600	550
Possible iron potential based on remaining precursors (ppm)	2800	1900	1400	150
Total iron potential (ppm)	3300	2500	2000	700

Conclusions Regarding Ageing Methods for Preserving Iron Losses

The data in Table III demonstrate that the total iron potential in orris rhizomes decreases during ageing while the actual iron content remains stable after a one-year ageing. We believe that precursors are slowly transformed into irones while at the same time these irones are lost through evaporation. A modification of the traditional ageing process in order to accelerate iron generation while reducing the evaporation rate speeds the production process and yields and orris essential oil with a higher iron content.

Vetiver Oil and its Derivatives

Vetiver essential oil is obtained from dried vetiver roots (*Vetiveria zizanoides* Stopf.). (13) Only the underground part of the plant, composed of numerous fine rootlets bears essential oil. The overground part may serve as

thatching of rustic housing in the bush. Vetiver plants, due to their highly developed root system are also commonly used to counteract erosion in many tropical countries.

The composition of vetiver essential oil is extremely complex. According to recent studies, more than 170 constituents have been reported. Most of them possess a sesquiterpenic structure. (17) The ISO standard for oil of vetiver provides good criteria for assessing vetiver oil quality. (18)

The use of whole vetiver essential oil as an ingredient in a perfumery compositions is relatively small compared to its use in the form of fractions and derivatives. When whole vetiver oil is used it is typically of Bourbon origin due to its outstanding olfactive quality. There are 3 principal derivatives of vetiver oil:

- Vetiverol - a fraction containing mostly sesquiterpenic alcohols and exhibiting a tenacious sweet-woody odor.
- Vetivone - a fraction containing mainly sesquiterpenic ketones and presenting a very tenacious earthy odor.
- Vetiveryl acetate - a derivative which particularly praised by perfumers. It is comprised of acetylated esters of the vetiverol fraction. Vetiveryl acetate possesses a fresh-sweet woody odor.

In terms of quantities used globally, vetiveryl acetate is the most important derivative. Vetiverol is the second most used product, while vetivone remains the item with the smallest use. Considering these global applications, it is clear that a key driver of vetiver essential oil usage is its vetiverol or sesquiterpenic alcohol content.

Sourcing a New Vetiver Origin

The vetiver products family is very important for perfume ingredient suppliers either for isolated tailored fractions or for conventional vetiveryl acetates of various kinds. For production of these derivatives two classical geographical origins of vetiver essential oil were used: Indonesia (Java) and Haiti. (14,15,16) The vetiver oil of Java origin is generally very poor olfactory quality. It requires further rectification steps to yield good vetiveryl acetate. The vetiver oil of Haitian origin is unique for its good vetiverol content that makes it very attractive to fragrance ingredient processors as a starting material for the prized vetiveryl acetate.

The objective of this study was to investigate sources of vetiver production beyond the Haitian source that is frequently subject to speculation. Madagascar was chosen as a production site because of the great plantation opportunities there.

Analytical Characteristics of Vetiver Essential Oils

The ISO standard for vetiver essential oil describes the quality characteristics for the various known origins. (18) Vetiver oil of Haitian origin possesses a low acid content compared to oil from Java that is highly acid. The high acid value of oil sourced from Java is due to a high content of sesquiterpenic carboxylic acids. The gas chromatographic analysis of vetiver oil of Haitian origin reveals a great amount of alcohols and ketones, almost twice the content found in oil of Java origin. Constituents formed by the dehydration of sesquiterpenic alcohols during hydrodistillation are very high in Java essential oil.

Starting from this understanding of the differences between oils from Java and Haiti, this work was designed to

- first, compare the vetiverol potential of both Madagascar and Haiti vetiver roots.
- second, propose a non-degradative process to optimize the extraction of vetiverol from Madagascar vetiver roots.

Determination of Vetiverol and Vetivone Potential of Vetiver Roots

Three constituents of vetiver oil: GC quantified khusimol and isovalencenol, the two major sesquiterpenic alcohols, and alpha-vetivone, a characteristic sesquiterpenic ketone. See Figure 8 for structures. The analytical methodology was as follows: determine the response factors of the 3 constituents, then perform a non-destructive extraction of vetiver roots and finally, quantify the 3 constituents in the extract of vetiver roots. The content of the three constituents in Haitian roots were determined from both a non-distilled extract and an oil obtained by distillation. Distilled vetiver roots were also extracted to determine the level of recovery of the essential oil by the industrial Haitian process.

Vetiver roots from three different plantations in Madagascar were also tested by the same method in order to compare results to that obtained for Haitian roots. The results of the tests are detailed in Table IV.

The potential for producing roots that can yield oils high in alcohols and also ketones was very high for the Madagascar trial fields. The only drawback for the Madagascar vetiver oils is that all the Madagascar oils and extracts possess a high acid content. The high acid content is responsible for the very poor quality of Madagascar vetiver oil. The processing of Madagascar vetiver roots must take into account the impossibility of using direct steam distillation of vetiver roots, as it is too destructive.

Table IV. Compositional analysis of Vetiver Roots

Origin of the root	Khusimol %	Isovalencenol %	alpha-Vetivone %
Vetiver Haiti roots (non-distilled)	0.22	0.10	0.12
Vetiver Haiti roots (distilled)	0.027	0.008	0.011
Vetiver Madagascar roots (location M)	0.45	0.25	0.19
Vetiver Madagascar roots (location C)	0.70	0.36	0.29
Vetiver Madagascar roots (location A)	0.93	0.47	0.30

Maximizing Vetiverol Content from Madagascar Vetiver Roots

A three-step process was chosen for maximizing the Vetiverol content of Madagascar vetiver roots. The process (Figure 7) included a neutral volatile solvent solid-liquid extraction, followed by removal of the sesquiterpenic acids by means of a liquid-liquid acid-base extraction. The third and final step was a short-path high-vacuum distillation to isolate acid-free vetiver oil. A comparison of yields and acid values for Madagascar and Haiti vetiver roots with this three-step process is presented in table V.

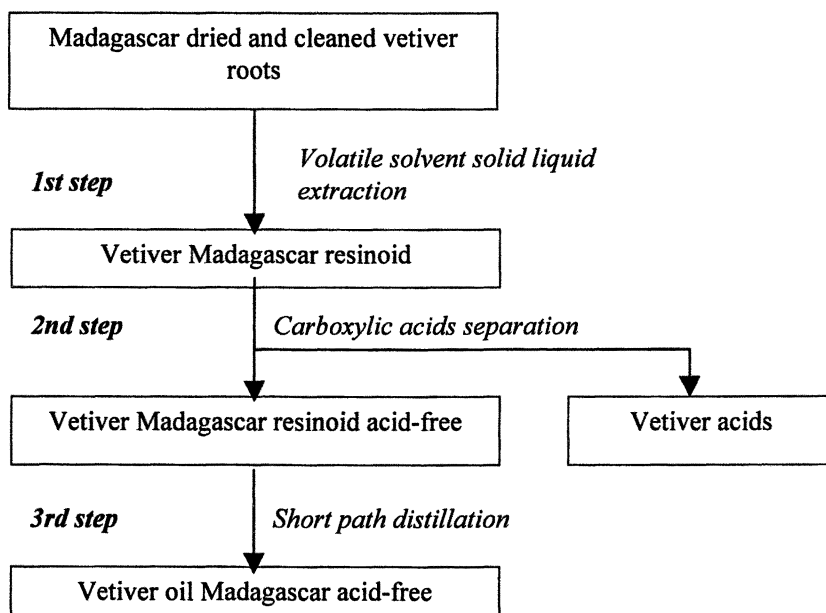
Table V. Comparative Analysis of Madagascar and Haitian Vetiver Roots

Vetiver root origin	Acid value after a simple resinoid distillation	Acid removal yield %	Total yield % from vetiver root	Final acid value of acid free vetiver oil
Madagascar	97.3	48.2	1.9	1.1
Haiti	32.3	83.8	0.6	1.2

The comparison of both Haitian and Madagascar compositions after the three-step process is presented in Table VI.

Table VI. Comparative Analysis of Madagascar and Haitian Vetiver Oils

Vetiver roots origin	Khusimol %	Isovalenceno 1 %	alpha vetivone %	beta vetivone %
Madagascar	11.5	11.1	8.4	5.1
Haiti	16.5	7.7	6.8	3.1

*Figure 7 Vetiver root processing flow chart*

Conclusions Regarding Optimizing Vetiver Oil Yield

Using the three-step process described above and in Figure 7 it is possible to obtain from Madagascar vetiver roots a very good yield of an alcohol-rich vetiver oil. The improvement in yield more than offsets the need to process the roots with a multi-step process.

The odor quality of this new oil is similar to that of vetiver oil from Haiti with a slightly smaller percentage of khusimol relative to isovalencenol. The joint

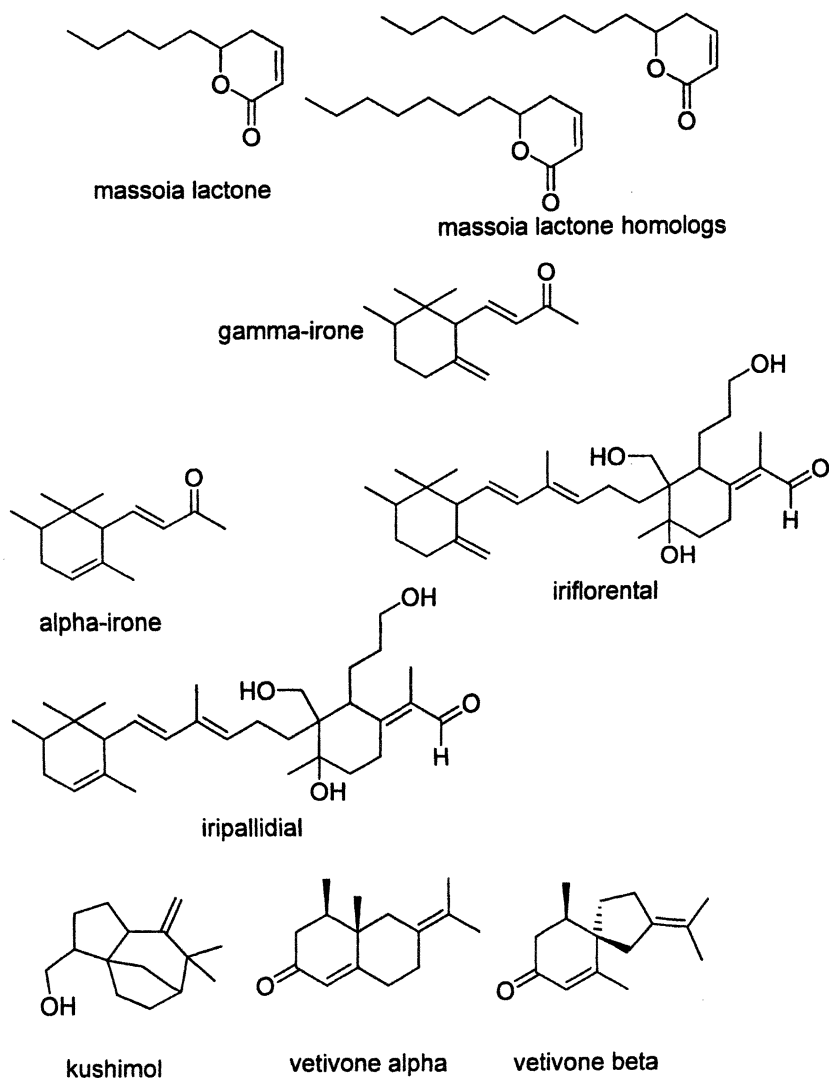


Figure 8. Important Components of Massoia, Orris and Vetiver

R&D and sourcing effort succeeded by obtaining an oil from Madagascar vetiver roots that could serve as a viable alternative to oil obtained Haitian vetiver roots

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References

- Gildmeister E, Hoffmann F., *Les Huiles Essentielles* 2nd edition by Gildmeister E. 1914, vol II p 549-551 for massoia bark essential oil; vol II p 503-504 for lawang essential oil.
- Guenther E, *The Essential Oils* D. Van Nostrand Company Inc. 1952, vol IV, p 211-213.
- Abe S., Massoi oil, *J. Chem. Soc. Japan* 1937, 58, 246-251.
- Meijer T. M., The Essential Oil of Massoia Bark, *Rec.Trav.Chim. Pays-Bas*, 1940, 59, 191.
- Abe S., Sato K., The Essential oil of Massoia bark III - Structure of Massoia Lactone, *J. Chem. Soc. Japan*, 1954, 75, 952-953.
- Cavill G.W.K., Clack D.V., Whitfield F.B., *Austr. J. Chem*, 1958, 21, 2819-2823.
- Garnero J., Joulain D., Massoia Essential Oil, *9th International Congress of Essential Oils*, Singapore (1983).
- Bernreuther A., Lander V., Huffer M., Schreir P., Enantioselective Analysis of Dec-2-en-5-olide (Massoia Lactone) From Natural Sources by Multidimensional Capillary Gas Chromatography, *Flavour and Fragrance Journal* 1990, 5, 71-73.
- Guenther E., *The Essential Oils* D. Van Nostrand Company Inc. NY (1952), vol IV, p 79-105.
- Krick W., Marner F.J. and Jaenicke L., Isolation and Structure Determination of the Precursors of α - and β - Irone and Homologous Compounds from *Iris pallida* and *Iris florentina*. *Z. Naturforsch*, 1983, 38c, 179-184.
- Jaenicke L., and Marner F.J., The Irones and Their Precursors, *Prog. Chem. Org. Nat. Prod.*, 1986, 50, 1-25.
- Bicchi C., Rubiolo P., Fresia M., Analysis of Constituents of Iris Rhizomes Part III - Packed Column Supercritical Fluid Chromatography and High Pressure Liquid Chromatography of Iridals from Rhizomes of *Iris pallida* L., *Phytochemical analysis* 1996, 7, 37-41.
- Guenther E., *The Essential Oils* D. Van Nostrand Company Inc. NY 1952, vol IV, p 156-180.

14. Smadja J., Gaydou E.M., Peyron L., Lamaty G., Conan J.Y., Etude Comparative D'huiles Essentielles de Vétyver D'origine Géographique Diverse, *Rivista Italiana EPPOS*, 1990, 1, 7-21.
15. Smadja J., Mise au Point Bibliographique sur L'huile Essentielle de Vétyver, *Rivista Italiana EPPOS*, 1994, December, 14, 15-36.
16. Peyron L., Le Vétyver et sa Culture dans le Monde, *Rivista Italiana EPPOS*, 1995, December, 17, 3-18.
17. Weyerstahl P., Marshall H., Splittgeiser V., Wolf D., 1,7-Cyclogermacra-1(10),4-dien-15-al, a Sesquiterpene with a Novel Skeleton, and Other Sesquiterpenes from Haitian Vetiver Oil. *Flavour and Fragrance J.*, 2000, 15, 61-83.

Chapter 3

1,1,1,2-Tetrafluoroethane (R-134a): A Selective Solvent for the Generation of Flavor and Fragrance Ingredients

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Originally developed as a refrigeration fluid for domestic appliances and automotive air conditioning applications, 1,1,1,2 tetrafluoroethane (R-134a) may also be used to good effect for the extraction of natural flavor and fragrance materials. This paper will explore the solvency characteristics of R-134a which, when combined with its convenient physical properties, make it a useful, viable alternative to conventional organic solvents and, in some cases, supercritical carbon dioxide.

Extraction of natural materials in order to produce extracts with useful flavor, fragrance, nutritional and other pharmacological properties is a centuries old practice. Traditionally, such extracts are isolated from the relevant biomass (flowers, roots, peel, leaves etc.) using a variety of processes ranging from steam distillation through solvent extraction to simple mechanical expression. Many of these initial materials have been subjected to further refinement steps in order to produce a finished product of acceptable quality.

Despite the length of time such processes have been used, there is an ongoing desire for the creation of new and fresher flavors and fragrances made with extracts that are closer in character to the wide range of new and existing raw materials from which they are derived. There is also significant interest in the isolation and identification of pharmacologically active materials derived from plant and marine organisms which may be used as a basis for drug or pesticide development. However, this search for new or improved ingredients has to be continually tempered by the cost of isolating these materials at any commercially significant scale.

Conventional extraction techniques are not able to produce new extracts with an acceptable quality/cost ratio in every case. Many of the traditional organic solvents are under increasing regulatory pressure due to the toxicity of residual solvent or are unacceptable to large groups of end-users for religious or other reasons. Many of these solvents also show poor selectivity and hence the initial products may require significant downstream processing which may actually dominate the costs associated with obtaining the final product. Conventional steam distillation can adversely affect the character of certain products through hydrolysis, thermolysis and loss of volatile or water-soluble top-note species that are vital to the development of the fresh character of the extract.

Supercritical carbon dioxide (scCO₂) extraction has been developed over the last couple of decades as a selective solvent extraction technology capable of generating very high quality products with a distinctive character, often quite unlike those obtained by conventional processes. The ability to independently control the pressures and temperatures used for both the extraction and product recovery steps allows the overall process to have a good degree of control over selectivity and recovery. While scCO₂ can produce high quality extracts with minimal solvent residues, the capital costs associated with building equipment rated to cope with the high pressures used, typically 100 bar to 500 bar, can result in prohibitively high product costs. In order to reduce this equipment cost impact, scCO₂ extractions can be conducted at large throughputs to achieve economies of scale. Examples of large-scale scCO₂ extraction processes include decaffeination of tea and coffee and extraction of hops for use in the brewing industry.

There is clearly scope in the market for a new extraction process, yielding cost effective, high quality extracts at a wide range of application scales.

This paper is intended to outline the properties of the hydrofluorocarbon solvent 1,1,1,2-tetrafluoroethane (R-134a) that make it an attractive proposition for the extraction of natural products and to highlight some of extracts that may be obtained.

1,1,1,2-tetrafluoroethane (R-134a)

Prior to the 1980s, the principal classes of chemicals used as refrigerants in the refrigeration industry were the chlorofluorocarbons ("CFCs") and the hydrochlorofluorocarbons ("HCFCs"). CFCs consist of molecules containing only atoms of chlorine, fluorine and carbon. HCFCs consist of molecules containing atoms of hydrogen in addition to atoms of chlorine, fluorine and carbon. In addition to their use as refrigerants, many of the CFCs and HCFCs were used as extensively as industrial and consumer product aerosol propellants, foam blowing agents and as solvents. Some, such as dichlorodifluoromethane (R-12), trichlorofluoromethane (R-11) and dichlorotetrafluoroethane (R-114) had been proposed as solvents for the extraction of natural products.

By the time of the Montreal Protocol on Substances that Deplete the Ozone Layer (the "Montreal Protocol"), it was widely recognised that chlorine containing refrigerants, particularly CFCs, were implicated in the depletion of the Earth's ozone layer. With the establishment of the Montreal Protocol and the subsequent legislation passed in participating countries, there came a need in the refrigeration industry to find suitable replacements for CFC refrigerants as quickly as possible. HCFCs were subsequently added to the list of substances to be phased out under the Montreal Protocol in 1992.

Hydrofluorocarbons ("HFCs") consist of molecules containing only atoms of hydrogen, fluorine and carbon and were known at the time of the Montreal Protocol to have effectively zero ozone depletion potential (ODP). Following the Montreal Protocol, many participants in the refrigerant manufacturing and end-user industries therefore looked towards HFCs as replacements for CFCs across a range of applications. R-134a is one such HFC.

R-134a has been known for many years and was identified as a potential refrigerant as long ago as 1934. From its known physical properties, R-134a was seen primarily as an alternative to R-12, the major refrigerant employed in automotive air conditioning and, on a smaller scale, in domestic appliances. R-134a is now manufactured at large scale in the US, Europe and Japan in order to supply these, and other, refrigeration applications across the globe.

General Physical and Chemical Properties of R-134a

R-134a is a non-flammable gas at room temperature and pressure with a normal boiling point of around -26°C . It is normally handled as a compressed gas under pressure in liquid form and has a liquid density of about 1300 kg/m^3 . With an extraction equipment design pressure requirement of only 20 bar or so, standard stainless steel fabrication techniques can be used with a significant cost saving, when implemented at industrial scale, over the more specialist techniques and materials required for operation at pressures greater than the 100 bar or so required for scCO_2 .

Although supercritical fluids offer the capability to control the solvent selectivity or strength through control of the fluid density, this requires control of both the process temperature and pressure in order to achieve reproducible processing. Being a saturated liquid governed by equilibrium, R-134a needs only its temperature or pressure to be controlled in order to control the conditions of extraction or product isolation. This allows far simpler control of the process.

R-134a is immiscible with water and glycol solvents, is sparingly soluble in water (1500 ppm by weight at 20°C @ 1 bar) but is miscible with a number of organic solvents including the lower alcohols, ethers esters and hydrocarbons. R-134a is extremely resistant to hydrolysis and in solution, has no effect on the pH of aqueous media. This is in contrast to CO_2 where significant reductions in pH are observed when pressurized CO_2 is in contact with water-rich biomass materials. R-134a can therefore be used to good effect to extract hydrolytically sensitive materials from aqueous solutions or from water-rich, fresh biomass materials.

Other physical properties of relevance to the use of R-134a as a solvent are viscosity and surface tension (Table I). In comparison to the conventional liquid solvents, R-134a has both low viscosity and low surface tension, promoting rapid solvent wetting and penetration of a solute matrix.

Approval Status

R-134a is not a volatile organic compound (VOC) and has no ozone depletion potential. Despite being both volatile and organic, R134a is not classified as a VOC under any of the Clean Air legislation. Its chemical stability is high enough that it is taken into the upper atmosphere without any generation of ground-level ozone (unlike most other organic solvents and hydrocarbons). It is only broken-down photochemically in the upper atmosphere by the higher-energy UV present there. R-134a is available at high purity and to cGMP standards for use as a medical propellant for inhalation applications (MDI) and

Table I. Viscosity and Surface Tension

Solvent	Viscosity (cP @25°C)	Surface Tension (Dyne/cm)
scCO ₂ (200bar, 33°C)	0.1	N/a
R-134a	0.21	8.7
Diethyl ether	0.223	17.0
Hexane	0.294	18.4
Acetone	0.316	23.7
Chloroform	0.53	-
Methanol	0.56	22.6
Water	0.89	73.0

Data from references 1 to 3.

now for food product extraction. In terms of toxicology, R-134a is one of the most extensively studied fluids with significant programs conducted prior to its introduction as a refrigerant and then as a pharmaceutical metered dose inhaler propellant. The first of these studies, the Program for Alternative Fluorocarbon Toxicity testing (PAFT 1) was a cooperative research effort over the period 1987 to 1990 involving 17 main sponsoring companies from 9 countries. R-134a was found to be of low toxicity in all of the studies. In reflection of this, R-134a is widely accepted as an extraction solvent for food materials. FDA "GRAS" notification of food grade HFC-134a was completed with no adverse comment. R-134a is approved for food extraction use under the European Directive on Extraction Solvents Used in the Production of Foodstuffs and Food Ingredients and a range of R-134a flavor extracts are already approved on an extract-by-extract basis in Japan.

R-134a As a Solvent

There are many ways of describing the behavior and solvency characteristics of fluids towards a given solute. Most practicing chemists are familiar with the solvency behavior of a number of mainstream organic solvents having developed a "feel" for the character of the solvent through their own experience. The old maxim of like-dissolves-like is one of the earliest rules in chemistry and still provides valuable guidance in the choice of solvent for a particular solute. Does this suggest that solvency applications or uses for R-134a should lie within the area of halogenated, especially fluorinated, solutes? The high solubility of R-134a in Viton™ and other fluoropolymers may be

consistent with this but if this were the case then the scope for application of R-134a as a solvent would be relatively narrow. As we will see below, this is clearly not the case.

From its chemical structure and general physical properties described above, R-134a can be regarded as a non-aqueous, aprotic, non-flammable halogenated fluid, stable to both aqueous acids and bases but in order to develop a quantitative or semi-quantitative view of the solvency behavior of R-134a, we need to look in more detail at its physical properties.

Table II. Solvent Dielectric Constant and Dipole Moment

Solvent	ϵ (1kHz)	DM
R-134a	9.5	2.05
Hexane	1.9	0.08
Diethyl ether	4.34	1.52
Ethyl acetate	6.02	1.83
Dichloromethane	9.08	1.55
Tetrahydrofuran	7.61	1.63
Acetone	20.7	2.9
Methanol	32.6	1.66
Water	78.3	1.8

Data from references 1 to 3.

Table II gives some values for the dielectric constant and dipole moment of R-134a and a of number of conventional solvents and scCO₂). R-134a is clearly more polar than several of the conventional solvents such as diethyl ether and tetrahydrofuran, having a higher dielectric constant and a higher dipole moment, both normally regarded as measures of solvent polarity. Supercritical carbon dioxide (scCO₂), being quadrupolar, is regarded as being a non-polar solvent over a wide range of operating pressures in the absence of polar modifiers. From a dipole moment and dielectric constant perspective, R-134a is not too dissimilar to dichloromethane, both fluids containing a methylene group attached to two electron-withdrawing groups. However, the behavior of R-134a as a solvent is quite dissimilar to that of dichloromethane. This is perhaps not surprising since the dipole moment and dielectric constant are only indications of what the chemist normally regards as solvent polarity. In practice, the interaction between the solvent and a solute cannot be accurately described by any one or two simple physical constants and is composed of many specific and non-specific contributions. This will be discussed in more detail below.

Cohesive Energy Approaches

The energy required to expand one mole of a fluid from its original state to infinite volume at constant temperature is the cohesive energy of the fluid. This is a direct measure of the strength of the intermolecular forces between the fluid molecules and for a liquid, can be broken down into two contributing steps; vaporization of the liquid to its saturated vapor followed by expansion of that vapor to infinite volume;

$$-\Delta U = \Delta H_{\text{vap}} + \Delta H_{\text{exp}} - RT + PV$$

where P is the saturation pressure at temperature T and V is the liquid molar volume. For supercritical fluids, which have no heat of vaporization, only the latter term applies. It has been found that a more useful measure of the cohesive effect in liquids is given by the cohesive energy density, or cohesive pressure, c ;

$$c = -\Delta U/V$$

the cohesive energy per unit volume of solvent. The cohesive pressure was used by Hildebrand and Scott as the basis of the Hildebrand solubility parameter, δ ;

$$\delta = c^{1/2}$$

The Hildebrand parameter can be determined experimentally by literature techniques but is especially straightforward for volatile solvents where heats of vaporization are available. The Hildebrand solubility parameter can be thought of as a crude measure of solvent “strength”, higher values indicating stronger or more numerous intermolecular interactions within the liquid phase of the substance. This interpretation of the significance of δ is over-simplistic but is of some value when dealing with mixtures of like species such as those within a homologous series where the nature and magnitude of the intermolecular interactions between species in the mixture are comparable to those present within the pure mixture components. Numerous works have dealt with the relationships between the Hildebrand parameter and relationships derived from it, and a wide range of solvent-related applications, particularly polymer-solvent systems but also including those of refrigerant species (see (4) for further description and discussion). In brief, liquids having similar Hildebrand parameters often have a high degree of mutual solubility. In many respects, this can be considered as the thermodynamic equivalent of “like-dissolves-like” and as mentioned above, can be a useful, but by no means universal, guide.

The Hildebrand parameter for R-134a calculated as a function of temperature using a Peng-Robinson equation of state is shown in Figure 1 along with values for a number of other conventional organic solvents and for scCO₂. At normal ambient temperatures, the Hildebrand parameter for R-134a can be seen to be towards the bottom of the range of values for most of the conventional organic solvents. The Hildebrand parameter of dichloromethane is 20.3 MPa^{0.5} at 25°C, close to that of acetone and considerably higher than that of R-134a. This, to a large degree, helps explain the difference in solvency behavior between the two fluids despite their similar dielectric constants and dipole moments as described above. The Hildebrand parameter for scCO₂ varies with fluid density (pressure) and is close to that of R-134a only at relatively low pressures, around 100 bar or so. The Hildebrand parameter for R-134a can be seen to increase as the temperature of the fluid is reduced. This is likely to be a reflection of the polarity of R-134a with the relatively weak directional polar interactions in the fluid being increasingly disrupted by thermal effects as the fluid temperature increases. Even at reduced temperatures, it can be seen that the Hildebrand parameter for R-134a remains below that of liquid CO₂, a selective solvent that is used commercially to obtain high quality natural product extracts.

In an attempt to improve on the Hildebrand parameter and take into account the range of intermolecular interactions present in the majority of real fluids, there have been a number of multi-contribution derivations of the Hildebrand parameter (4). Probably the best known of these are the Hansen parameters where the Hildebrand parameter is broken down into three intermolecular contributions - dispersion forces, polar forces and hydrogen bonding forces;

$$\delta^2 = \delta_D^2 + \delta_P^2 + \delta_H^2$$

Table III. Hildebrand and Hansen Parameters (MPa^{0.5})

Dispersion	Polar	H-Bond	Hildebrand
7.2 to 8.4	7.4 to 8.9	7.5 to 9.4	13.8

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A range of Hansen parameter values have been reported for R-134a and are shown in Table III. This variety in Hansen values is not unusual since by their nature, there is no direct method of measuring them. The values in Table III are in broad agreement and show that R-134a has similar contributions from each of the three interaction types. This can be illustrated graphically in Figure 1, where the fractional Hansen parameters for R-134a and a range of conventional solvents are shown in map form on a triangular diagram. The fractional Hansen

parameter is the ratio of the individual Hansen parameter to the total Hildebrand value. From its position on the Hansen map, R-134a is perhaps most like acetone in terms of its balance of contributions but has a distinctly different Hildebrand parameter.

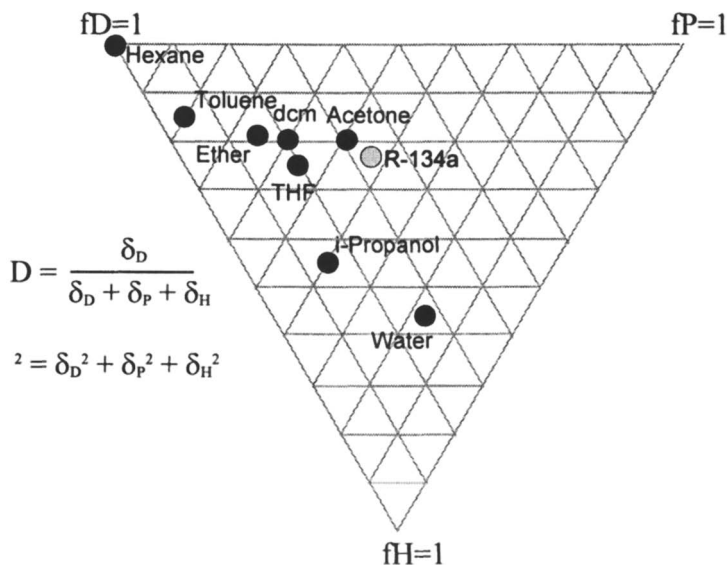


Figure 1. Fractional Hansen parameter map

In mixtures where there is limited mutual solubility, due in large to a significant difference in Hildebrand parameters, it is often the case that the non-specific solvent-solute interactions as exemplified by the dispersion Hansen parameter, govern the behavior of the system (4). This can be readily rationalized on the basis that species displaying high cohesive energies have significant specific (polar and hydrogen bonding) interactions while those with low cohesive energies exhibit considerably weaker specific interactions. The resulting interactions between a substance with a high cohesive energy and one with a low cohesive energy will be dominated by the mode of interaction common to both, the non-specific dispersion forces embodied in the dispersion Hansen parameter. We have already seen that the dispersion Hansen parameter of R-134a is low in comparison with most conventional organic solvents suggesting that R-134a will not act as a powerful solvent across a wide range of solute types, unlike dichloromethane, for example.

We can graphically illustrate the character of solvents by plotting their dielectric constant, a measure of polarity, against the dispersion Hansen parameter, a measure of the non-specific solvent-solvent forces available for solvation. From Figure 2, it can be seen that R-134a is moderately polar but with a relatively low dispersion Hansen parameter, comparable to that of low pressure scCO₂. We might expect that the relative position of two materials on this map may be related to their mutual solubility to some extent, substances that are further apart having the lowest mutual solubility (another example of the like-dissolves-like principle). For example, a hydrocarbon wax that may be located in the lower right part of the diagram is more likely to be soluble in hexane than in ethyl acetate and more soluble in high pressure scCO₂ than in lower pressure scCO₂, both in keeping with our general experience.

On this basis, we might expect R-134a to have low solubility for a wide range of solutes with high dispersion Hansen values but within that group of solutes, R-134a would preferentially dissolve the more polar species over the non-polar species. It is this behavior that contributes to the attractive solvency character of R-134a for extraction of flavor and fragrance materials where many of the substances of interest contain one or more polar heteroatom groups.

Solvatochromic parameters

A problem common to all of the cohesive energy approaches to solvent characterization is that they based on the properties of the pure solvent and of the pure solute in isolation. These approaches are not readily able to deal with situations where there are significant specific solvent-solute interactions that are not exhibited in either pure component. In effect, the solvent, or solute, is assumed to only be capable of interaction with other species in the same way that it interacts with itself. A more widely applicable approach to would takes into consideration the potential for specific solvent-solute interactions.

One such methodology is the Kamlet-Taft Solvatochromic parameter approach. In this methodology, a solvent can be characterized by three parameters, π^* , a measure of the polarity and polarizability of the fluid, α , the acidity or hydrogen bond donor capability and β , the hydrogen bond acceptor capability or basicity. Each of these parameters is determined from the shift in UV-visible absorbance of a series of select indicator species dissolved in the solvent. Rather than depending on the bulk properties of the fluid, as is the case with the cohesive energy approaches, the solvatochromic parameters are derived from the interactions between the indicator solute and the immediate solvent shell, in effect they are a measure of how a solute “sees” the solvent. In each case, the scale of values has been normalized to between 0.0 for cyclohexane

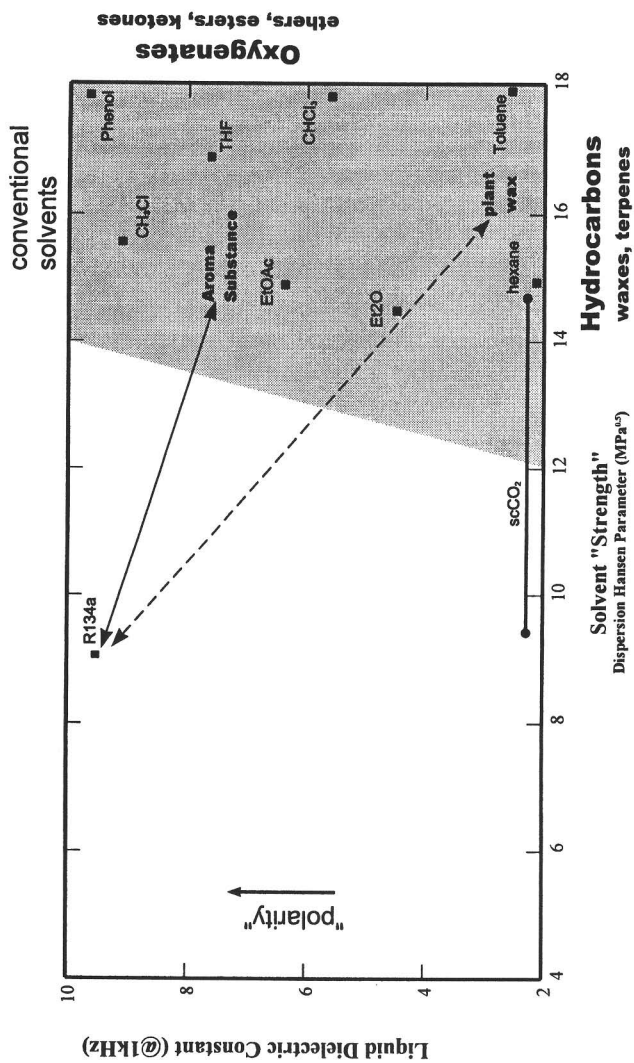


Figure 2. Polarity-dispersion Hansen parameter map
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and other hydrocarbons, to around 1.0 for an appropriate solvent for each property, for example dimethylsulphoxide (π^*), methanol (α) and hexamethylphosphoric acid triamide (β).

From the values of the Kamlet-Taft solvatochromic parameters (Table IV), R-134a is seen to behave as a moderately polar, weakly polarizable fluid with little or no basicity and weak acidity (of the order of dichloromethane). The negative value for β has been commented on previously (5) and may be an artifact of the original scale definition, the regression for which did not include heavily fluorinated species. In any case, the value suggests that R-134a is a poorer hydrogen bond acceptor than, for example, hydrocarbons.

The moderate α value for R-134a would suggest that R-134a should have a favorable interaction with solutes containing basic functional groups. Examples of such functional groups include ethers, esters, alcohols, ketones and amines which, coincidentally, are the majority of the functional groups found in many of the key flavor and fragrance materials.

Having looked at some of the solvent characteristics of R-134a, it is clear that R-134a exhibits an interesting balance of solvent properties. This balance of properties makes it difficult to equate the behavior of R-134a with any single conventional organic solvent. Does the combination of its physical and solvency properties bring any advantages over conventional organic solvents and scCO₂?

Table IV. Solvent Solvatochromic Parameters

Solvent	π^*	α	β
R-134a	0.27	0.48	-0.14
scCO ₂	-0.2 to 0	0	0.0
Cyclohexane	0.0	0.0	0.0
Diethyl ether	0.27	0.0	0.47
Ethyl acetate	0.55	0.0	0.45
Dichloromethane	0.82	0.30	0.0
Tetrahydrofuran	0.58	0.0	0.55
Acetone	0.72	0.08	0.48
Methanol	0.60	0.93	0.62
Water	1.09	1.17	0.18

Natural Product Extraction

It has been found that R-134a can be used to extract a range of useful products from a wide range of materials of natural origin. Products that may be extracted include natural flavors and fragrances and nutraceutical extracts. The use of R-134a as the extraction solvent can address many of the shortcomings of existing isolation processes, including scCO₂. Extraction can be conducted anywhere within the liquid temperature range of R-134a but is normally conducted around or below normal room temperature. Because of the high volatility of R-134a, product recovery from the solvent can be conducted efficiently at relatively low temperature, minimizing the loss of volatile top-note components from the extract while still achieving minimal solvent residues in the isolated product. Throughout the extraction and recovery process, the raw materials and extracts are not subjected to elevated temperatures, minimizing product thermal degradation, and are maintained in an anaerobic environment, minimizing the effects of oxidation and rancidity. With the only water present being that found within the raw material itself and with no acidity as a result of CO₂ dissolution in that water, hydrolytic or acid-catalyzed degradation of any sensitive materials present is also minimized.

We can now look at some illustrative extracts obtained using R-134a. In general terms, the extracts obtained by R-134a extraction are of high intensity as a result of the retention of the more volatile top-note species and a high concentration of "active" species with little or no dilution by inert lipid and wax materials. As a result of the relatively solubility of waxes, lipids and resinous materials in R-134a, the extracts obtained generally require little or no post-extraction refinement processing in order to make them compatible with a number of application carrier fluids such as ethanol. While having a high content of the more volatile components, the R-134a extracts are not simply analogous to volatile oils. The extraction process still brings out many important low volatility materials resulting in an extract that is often somewhere between an essential oil and an oleoresin in character.

Ginger Extract

This intermediate essential oil/oleoresin character is apparent in R-134a ginger extracts. The product, obtained in around 4% yield from dried Nigerian root, is a clear, mobile golden-yellow oil. By preserving the citral content of the raw material as well as the major sesquiterpenic components, the R-134a extract has a warm, spicy ginger aroma with a distinctive fresh lemony undertone.

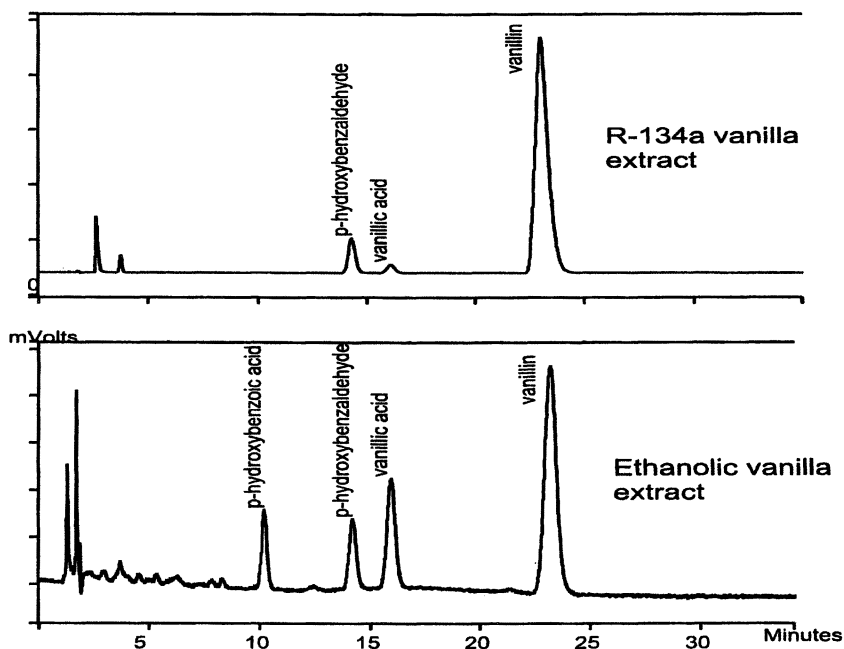


Figure 3. *Vanilla bean extracts HPLC*
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From a flavor perspective, the extract contains good levels, around 15% of the extract, of the gingerols and shogaols responsible for the heat component of the flavor resulting in a warm, fresh ginger flavour. Vanilla Bean Extract

Vanilla Extract

The R-134a Madagascar Bourbon bean extract is a creamy-yellow colored, semi-crystalline paste with a characteristic beany-vanilla odor, very similar to that of the original bean. The R-134a extract has a typical yield of around 7% and has around 90% vanillin recovery from the bean. While the vanillin recovery is high, R-134a does not produce an extract with the same composition of major flavor/fragrance components as that of a conventional ethanolic extraction. This is highlighted in Figure 3 which shows the HPLC analysis of the R-134a and ethanolic extracts of the same Madagascar Bourbon bean. The R-134a extract has significantly lower levels of vanillic acid and little or no p-hydroxybenzoic acid yet has good levels of p-hydroxybenzaldehyde. Despite, or perhaps because of, the low levels of these particular acids, the R-134a extract presents an extremely creamy vanilla flavor.

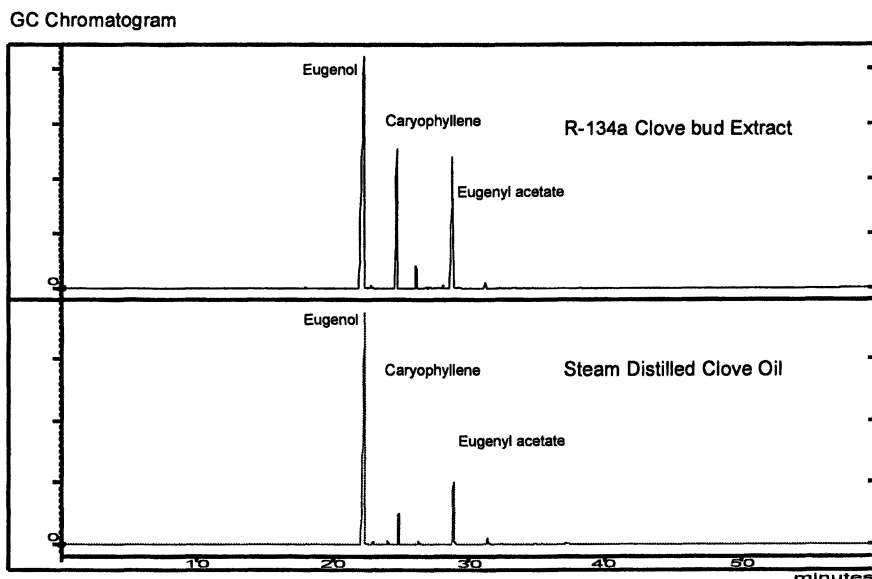


Figure 4. Gas Chromatographic comparison of Clove Bud extracts

Clove Bud Oil

The R-134a clove bud extract has a smooth, spicy clove aroma with carnation and rich plummy undertones derived from the high levels of caryophyllene (around 20%) and eugenyl acetate (around 30%) present in the extract. This is in contrast to a traditional steam distilled product which is relatively harsh and is dominated by eugenol with only around 4% eugenyl acetate and low levels of caryophyllene. The scCO₂ products are generally intermediate in composition between the R-134a extract and the distilled oil but still suffer from a degradation of the relatively sensitive eugenyl acetate component. As discussed earlier, the relatively low pH of wet CO₂ systems may result in some hydrolytic degradation of materials such as eugenyl acetate. A typical gas chromatograph of the R-134a extract and commercial steam distilled products are shown in Figure 4.

Floral Concretes

Floral concretes are the waxy solids derived from the extraction of fresh flowers with a solvent such as hexane or dichloromethane. R-134a can be used to good effect to generate ethanol-soluble absolute oils in one step directly from floral concretes, thus avoiding the multi-step process involving a series of solvent partitions normally required in order to manufacture an absolute from such a concrete. This application makes good use of the selectivity of R-134a for the small, polar fragrance components over the bulk of the waxy matrix of the concrete material. For example, R-134a extraction of Jasmine concrete yielded an intense sweet-floral-fruity yellow oil in good yield leaving behind a virtually odorless waxy residue. GC-MS analysis of the oil found the major volatile components; benzyl acetate, linalool, eugenol, cis-jasmone, indole and α -farnesene. R-134a has effectively extracted the desirable fragrance components, leaving the bulk of the undesirable plant waxes behind. Other floral concretes can be similarly processed.

Other Processes

In addition to the raw material extractions described above, R-134a can also be used to good effect in other processes of value in the flavor and fragrance sector such as reduction of the terpenic compound levels in citrus products in

order to produce “folded” oils. Another example of such a process is the removal of phototoxic furocoumarins species such as bergapten from natural oils such as bergamot. HPLC traces of pre- and post-treated Sicilian bergamot oil (Figure 5) clearly show the significant reduction in bergapten levels and coincidentally, the innocuous citropten. Unlike traditional methods of bergapten removal, there was little impact on the composition or fragrance of the treated oil.

Processing Technology

The extraction process is a closed-loop solvent circuit as illustrated in Figure 6. The biomass is contacted with liquid R-134a from a liquid receiver at a controlled temperature and solvent flow rate. The solvent, containing dissolved extract, is then transferred to a solvent evaporator where the solvent is vaporized and the extract is recovered. The solvent vapor from the evaporator is compressed and delivered at high pressure to a condenser where it is re-liquefied. The liquid solvent is then transferred to the liquid receiver, ready to repeat the cycle. This process is continued until the biomass is exhausted or until the desired extract is obtained.

The equipment has been designed with several economic and environmental performance criteria in mind. First, it needed to be capable of efficient and rapid recovery of product from the raw material. Second, it should have a low utility consumption through good energy efficiency and heat integration. Third, losses of solvent from the cycle should be minimized in order to minimize any global warming impact associated with the solvent and to minimize the costs of solvent replenishment. Finally, the equipment should provide product representative of full-scale production. Through a significant process design effort, these criteria have been met and the equipment, capable of extracting up to 100 tons of raw material per year, is shown in Figure 7.

Summary

Extraction with R-134a provides a new opportunity for the development of a cost-competitive range of high quality natural extracts having exciting product characteristics.

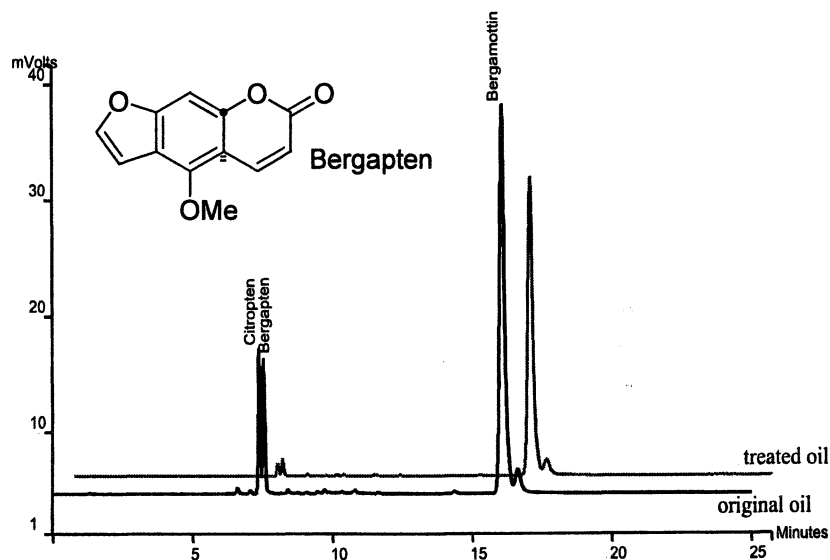


Figure 5. Removal of Bergapten from bergamot oil
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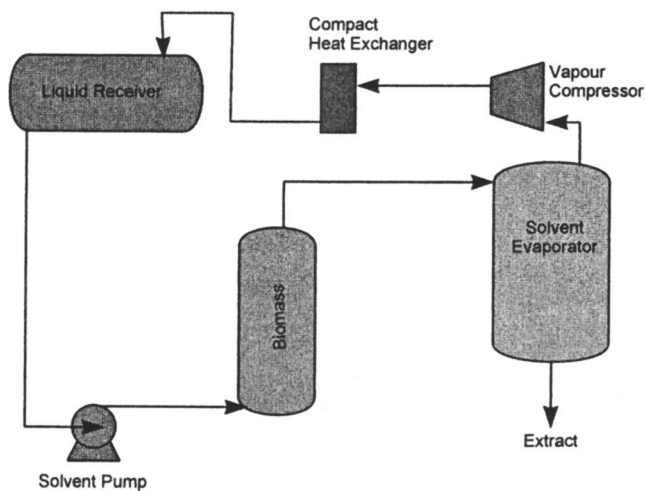


Figure 6. R-134a Extraction circuit schematic
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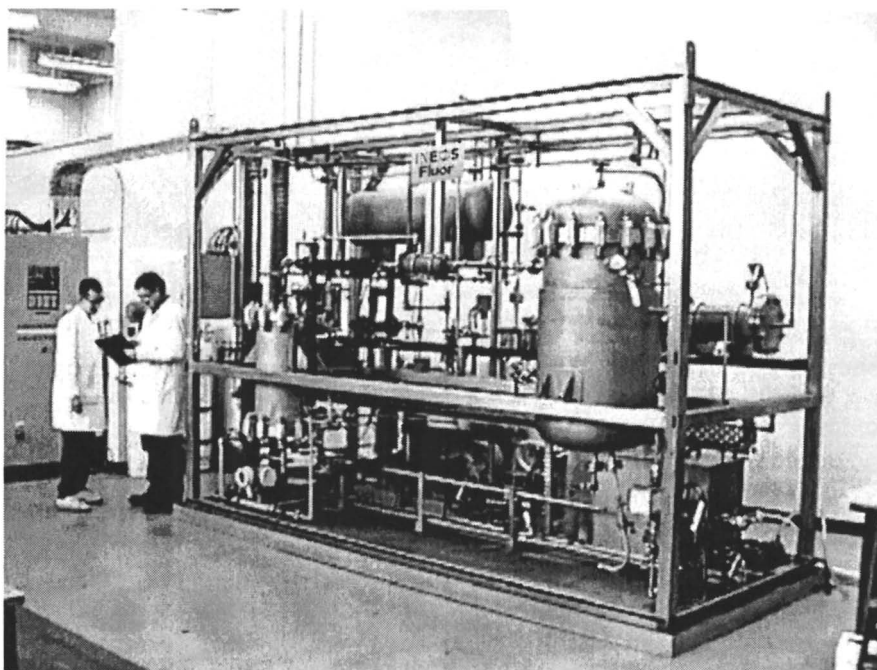


Figure 7. Small-scale R-134a extraction unit
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References

1. Corr, S. *J. Fluorine Chemistry* **2002** 118, 55
2. Weast, R.C. *CRC Handbook of Data on Organic Compounds*, CRC Press Boca Raton Florida, 1985
3. Reichardt, C.R. *Solvents and Solvent Effects in Organic Chemistry* 2nd Edition, VCH Basel, 1990
4. Barton, A.F.M. *CRC Handbook of Solubility Parameters and Other Cohesion Parameters* 2nd Edition, CRC Press Boca Raton Florida, 1991
5. Lagalante, A.F., Hall, R.L., Bruno, T.J. *J. Phys. Chem.* **1998** B, 102, 6601

Chapter 4

Application of Biotechnology to the Production of Natural Flavor and Fragrance Chemicals

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Most natural flavor/fragrance chemicals are heavily dependent on plant and animal origins. However, the quality and the supply of traditional natural flavor/fragrance chemicals are somewhat limited. Viable alternative and innovative ways to synthesize flavor and fragrance chemicals include biotechnological routes, i.e., microbial fermentation and plant tissue culture. Microorganisms are being used not only in the brewing and food industries to produce fermentation products, but also to produce aroma chemicals. The ability to produce aroma chemicals by microbial fermentation may supplement and enhance the quality of plant-based flavor/fragrance chemicals. Recent developments of commercialized processes to produce and/or to biotransform natural precursors into valuable flavor/fragrance chemicals via microbial metabolic pathways include the following; 1) Production of Tuberose lactone (a new GRAS chemical) via hydroxylation of unsaturated fatty acids and limited β -oxidation of the hydroxylated fatty acids; 2) Production of chirally active (R)-styrallyl acetate by regioselective reduction of acetophenone to styrallyl alcohol and subsequent esterification; and 3) de novo synthesis of chirally pure (+)-jasmonic acid and subsequent esterification to methyl jasmonate. Microbial biotransformation and biosynthesis of flavor and fragrance chemicals offer the potential benefits of producing optically active isomers which often have marked differences in flavor and fragrance quality and sensory intensity.

Introduction

Biotechnology can be applied in the flavor and fragrance industry in terms of the production of flavor and fragrance chemicals, of the production flavor and fragrance modulators and of the analysis of chemosensory systems. Probably, the production of natural flavor and fragrance chemicals is the most feasible way to apply biotechnology in the flavor and fragrance industry. Most natural flavor and fragrance chemicals are heavily dependent on plant and animal origins. However, the quality and the supply of traditional natural flavor and fragrance chemicals are somewhat limited. The supply of natural chemicals from plants is dependent on uncontrollable weather and sociopolitical instability of major supplying areas. Viable alternative and innovative ways to synthesize natural chemicals include biotechnological routes, i.e., microbial fermentation and plant tissue culture. Natural flavor chemicals especially, can be produced by fermentation, enzymology and natural reaction process under the regulation established by FDA (US Code of Federal Regulation, 21 CFR101.22.a3, 1990) (19).

Microorganisms are traditionally used not only to produce fermentation products, but also to produce aroma chemicals to enhance product quality in the brewing and food industries. The ability to produce aroma chemicals by microbial fermentation may supplement and enhance the quality of plant-based flavor and fragrance chemicals. The application of diverse microbial metabolic abilities is a major approach for the bio-production of flavor and fragrance chemicals in terms of feasibility and economics. A recent application of biotechnology in the flavor and fragrance industry is the production of natural chemicals using combinatorial approaches to develop environmentally benign bio-organic preparations of natural chemicals. The bio-organic processes, including whole cell fermentation, used in the commercial production of flavor and fragrance chemicals are summarized in Table I. Unlike chemical synthesis, bio-organic synthesis can have added benefit of producing optically active chemo-, regio- and stereo-selective chemicals in addition to the production of new natural chemicals. Such selectivity has marked effects on flavor and fragrance quality and intensity (16). The optically active and stereo-selective flavor and fragrance chemicals developed using biotechnology can be produced by; 1) de novo biosynthesis of pure isomers, 2) biotransformation of optically inactive precursors to optically active isomers, and 3) resolution of racemic mixtures to select optically active isomers.

The major flavor and fragrance companies are currently focused on biotechnological research efforts in discovery, identification and development of new flavor and fragrance ingredients to provide advantages in creating unique, globally natural, kosher, consumer preferred, high performance flavors and fragrances. The guiding principles drawn on by flavor and fragrance industries for the production of natural chemicals are the following: 1) source materials must occur in nature, 2) the resulting materials from processes must be found in

Table I. Commercial Bio-organic Processes used for the Production of Natural Flavor and Fragrance Chemicals

Chemical Reactions	Products
Oxidation of primary alcohols	Aldehydes and Acids
β -oxidation (aborted)	Methyl ketones
Hydroxylation (Oxidation) of aromatic acids	Hydroxyl aromatic acids
Hydroxylation of fatty acids	Saturated lactones
Hydroxylation & limited β -oxidation of fatty acids	Unsaturated lactones
Baeyer-Villiger oxidation	Alcohols
Reduction of Ketones	Secondary alcohols
Reduction of acids	Aldehydes and Alcohols
Reduction of double bonds	Saturated aromatic chemicals
Amino acid metabolism	Unsaturated & branched acids
<i>De novo</i> synthesis	Acids and alcohols

nature or in traditional foods, and 3) processing conditions must meet the criteria of traditional or in-home preparation techniques (20). In this paper, three newly commercialized bioprocesses for the production of, and or biotransformation of, natural precursors into new valuable flavor and fragrance chemicals via microbial metabolic pathways are discussed.

Tuberoso Lactone

Lactones are among the major ingredients used for the creation of flavor formulae in many categories, especially in fruit and dairy flavor formulae. Lactones are intramolecular esters that can be formed in plants and microorganisms. Over the years, a variety of saturated and unsaturated γ - and δ -lactones are produced by both chemical synthesis and microbial fermentation. The microbial processes for lactone production can be divided into two different processes; 1) the lactones can be produced by direct hydroxylation of fatty acids to produce d/l (+/-) 4- or 5-hydroxy fatty acids followed by a lactonization reaction, and 2) the lactones can be produced by two step processes of fatty acids, hydroxylation of unsaturated fatty acids and limited β -oxidation of hydroxy fatty acids to produce 4- or 5-hydroxy fatty acids followed by a lactonization reaction. Most commercial developed bioprocesses utilize microbial abilities to produce 4-hydroxy fatty acids for the production of γ -lactones. Various molds including *Aspergillus sp.* are known to directly hydroxylate at the C4-position of short chain fatty acids (8). Unlike the direct hydroxylation by molds, unsaturated fatty acids can be hydrated at different double bond positions, especially at the C10-position by various microorganisms (12). The resulting 10-hydroxylated fatty acids are converted to lactone intermediates, 4-hydroxy fatty acids, via limited β -oxidation of various yeasts for the production of saturated and or unsaturated γ -lactones. Although certain commercial fermentation processes have been well developed, the metabolic pathways for the accumulation of lactones in microorganisms are poorly understood (22). It has been speculated that the lactone intermediates, 4-hydroxy fatty acids, may cause structural hindrance for β -oxidation related enzymes and allow transient accumulation of intermediate 4-hydroxy fatty acids during β -oxidation processes of certain yeast cell cultures (8,22). The intermediates can be further oxidized to 3,4- and 3,5-dihydroxy fatty acids and eventually degraded to CO₂ and ATP as final products of β -oxidation beta during prolonged fermentation.

Natural and kosher tuberoso lactone is one of the lactones that was produced using a two-step fermentation and the a schematic process of tuberoso lactone is described in Figure 1. Tuberoso lactone, a mixture of saturated and unsaturated C12 lactones, was produced from hydrolyzed flaxseed oil. Flaxseed oil contains high concentration of C-18 unsaturated fatty acids (>90%),

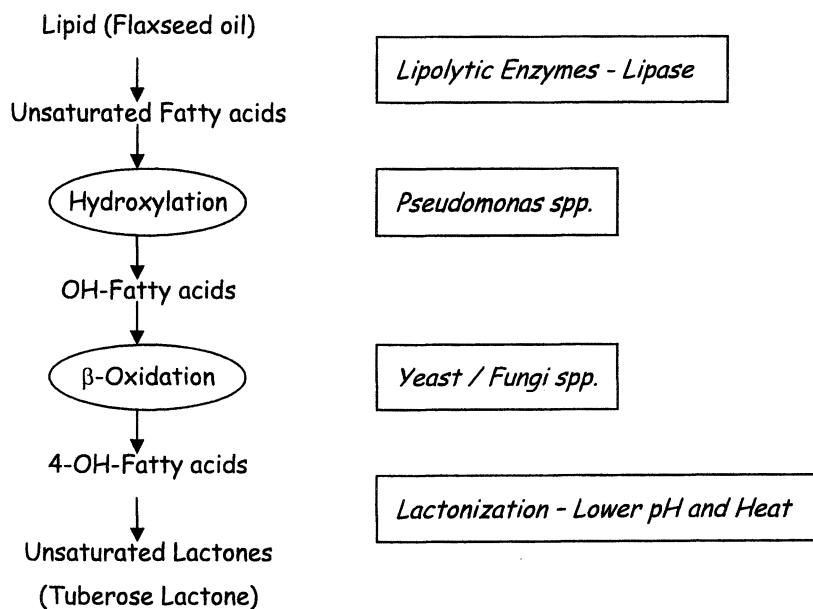


Figure 1. Schematic production flow diagram for the production of tuberoso lactone – Two-step fermentation process

including >50% linolenic acid, >20% linoleic acid and >20% oleic acid. Tuberoso lactone consists of three different lactones, (Z,Z)-6,9-dodecadien-4-olide (45-50%), (Z)-6-dodecen-4-olide (20-25%) and d-dodecan-4-olide (γ -dodecalactone, 20-25%), reflecting the composition of flaxseed oil (Figure 2).

The production requires 2 fermentations. In the first fermentation, lipase enzymes liberate the unsaturated fatty acids of flaxseed oil. During the first fermentation, the hydrolyzed fatty acids, linolenic acid, linoleic acid and oleic acid, are converted to (respectively) 10-hydroxy-12(Z),15(Z)-octadecadienoic acid, 10-hydroxy-12(Z)-octadecenoic acid and 10-hydroxydecanoic acid by *Pseudomonas sp.* NRRL-2994. *Pseudomonas sp.* produced stereochemically pure d (R)-isomers of each of the hydroxy fatty acids (>95.8%) (23) at a concentration of >12 g/L in the fermentation broth. The resulting hydroxy fatty acids were recovered by phase separation technique, and used for the second fermentation.

The second fermentation was developed using yeast strains, such as *Yarrowia lipolytica* ATCC 34088, that has limited β -oxidation abilities. The recovered hydroxy fatty acids were fed into a new fermenter and sterilized with other ingredients before inoculation with *Y. lipolytica* culture. *Y. lipolytica* converted C-18 10-hydroxy fatty acids to the corresponding lactone intermediates, 4-hydroxy C12 fatty acids via a limited β -oxidation. The fermentation was usually terminated at the point of a maximum accumulation of lactone intermediates, at the concentration of \sim 5 g/L in the fermentation broth. After the fermentation process was complete, the lactone intermediates were lactonized at a pH in the range of 3-5 and at a temperature of >100°C. The resulting lactones were recovered and purified from the fermentation broth by solvent extraction followed by fractional distillation.

Natural and kosher tuberoso lactone was recently listed in the GRAS 20 list of flavor chemicals. It is mainly used for the preparation of natural fruit and dairy flavors. They can also be used in various flavor and fragrance formulae as an enhancer or as a modulator due to their prolonged and tenacious sensory properties.

Styrallyl Acetate

Esters are among the major aroma chemicals in flavors and fragrances of all categories and can be synthesized by microorganisms and microorganism derived enzymes. However, microorganisms and enzymes exhibit substrate specificities that limit their application to the production of various esters. It is especially difficult to produce secondary alcohol esters using commercially available esterification catalyzing enzymes. The commercially developed process for secondary alcohol esters includes two separate processes: 1) the

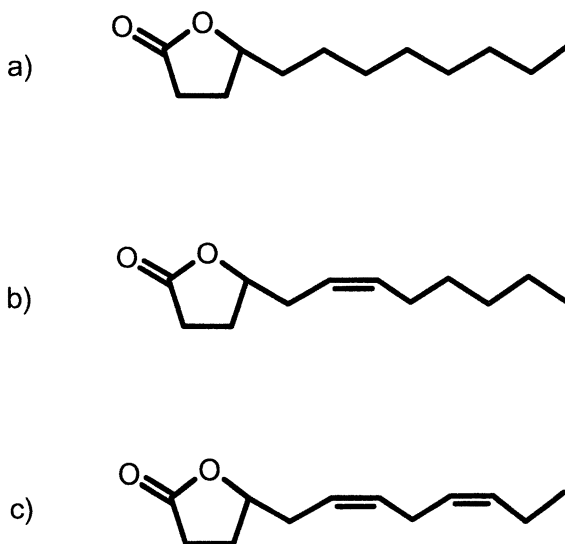


Figure 2. Tuberose lactone as a mixture of three different lactones: a) *d*-Dodecan-4-olide (20-25%), b) *d*-(*z*)-6-Dodecen-4-olide (20-25%), and c) *d*-(*z,z*)-6,9-Dodecadien-4-olide (45-50%)

microbial reduction of ketones to secondary alcohols, and 2) the esterification reaction of secondary alcohols with acetic acid under natural reaction conditions. The microbial reduction of ketones for the production of chiral secondary alcohols has been well known and used in the production of particular stereoisomers of specific secondary alcohols by the flavor and fragrance industry (Figure 3). The hydrogenation of ketones to form chiral secondary alcohols using *Clostridia* such as *Clostridium kluyveri* has been reported, and specially used in the production of styrallyl alcohol stereoisomers (17). Asymmetric reduction of acetophenone to styrallyl alcohol with enzymes of acetic acid bacteria was also reported by Adlercreutz (2).

Novel mixtures of optical isomers of natural and kosher styrallyl alcohol (α -phenylethyl alcohol), and their corresponding acetate esters of styrallyl alcohol (α -phenylethyl acetate) were prepared by multiple fermentation processes and an azeotropic esterification reaction. In the first step, natural acetophenone was produced by bioconversion of cinnamic acid by *Pseudomonas sp.* (9), *Comamonas sp.* and *Arthrobacter sp.* (6). In the first microbial oxidation process, the side chain of cinnamic acid was oxidized to the ketone to form acetophenone that was transiently accumulated in the fermentation broth (9). The current commercial fermentation process yielded >5g/L of acetophenone in the fermentation broth following 2 days of incubation using *Arthrobacter sp.* The resulting acetophenone was recovered and purified from the fermentation broth by solvent extraction followed by fractional distillation. Acetophenone itself can be used in creating flavor formulations and in enhancement of aroma and taste or both.

In the second step, a microbial process of reducing acetophenone to produce styrallyl alcohol stereoisomers was developed using a culture of *Kluyveromyces polysporus* ATCC 22028. *K. polysporus* quantitatively converted acetophenone to styrallyl alcohol at a concentration of >10 g/L in the fermentation broth following 2 days of incubation. The resulting styrallyl alcohol isomers (87% R- and 13% S-isomers) were recovered and purified by solvent extraction followed by fractional distillation. The crude styrallyl alcohol recovered from a short path distillation was used in the esterification reaction. Styrallyl alcohol itself also can be used in creating flavor and fragrance formulations.

Finally, the esterification reaction with styrallyl alcohol and acetic acid was carried out under natural reaction conditions using esterification catalysts such as citric acid or by means of bioprocess reactions using commercial ester-forming enzymes (5). The catalytic conversion of styrallyl alcohol to styrallyl acetate did not change the stereoisomer ratio. The resulting stereoisomeric mixture of styrallyl acetate was recovered and purified by solvent extraction followed by fractional distillation.

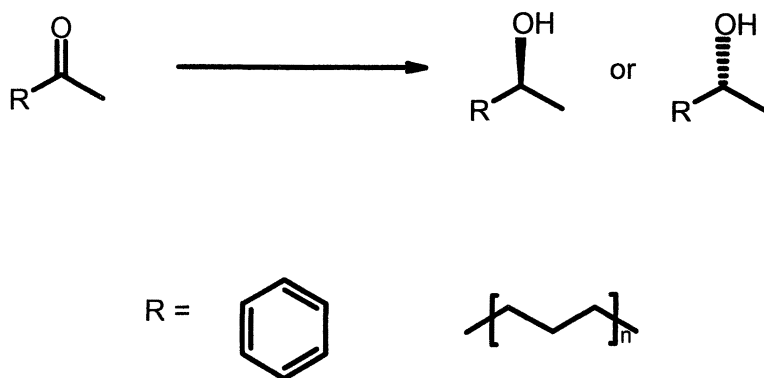


Figure 3. Ketone reduction by microorganisms. Ketones are selectively reduced to either S - or R -isomers

The overall bioprocesses produced a stereoisomeric mixture of natural styrallyl acetate with enantiomeric excess of 87% of the R-isomer. Styrallyl acetate mainly can be used for natural savory, fruit and dairy flavors, and fresh fruity and floral perfume compositions.

Methyl Jasmonate

Jasmonic acid (3-oxo-2-(2'-pentenyl)-cyclopentaneacetic acid), a plant fatty acid metabolite, is an endogenous plant growth regulator and is widely distributed in the plant kingdom (3). Jasmonic acid has various physiological functions including fruit ripening, production of pollen, root growth, tendril coiling, wounding, abiotic stress, and plant defense systems from microbial infection at lower concentrations in plants (3). Jasmonic acid is produced from the unsaturated fatty acid, linolenic acid, by plant lipoxygenase related metabolic pathways (Figure 4) and is probably a part of lipoxygenase related plant growth regulation (18,21). Its production starts with linolenic acid being converted to the 13-hydroperoxide of linolenic acid by lipoxygenase and further converted to the allene oxide by allene oxide synthetase. The allene oxide is metabolized to jasmonic acid via cyclization, double-bond reduction and side chain β -oxidation (21). It is also reported that several species of plant pathogenic fungi including *Diplodia sp.* produce jasmonic acid which show a phytotoxic effect at the relatively higher concentration (11). Although the biosynthetic pathway of microorganisms is probably similar to that of plants, the function of jasmonic acid in microorganisms is not clearly known. Jasmonic acid has two chiral centers at the C3 and C9 positions, and is expected to have potentially 4 stereoisomers (Figure 5) However, the absolute configuration of jasmonic acid in nature is not clearly known due to the difficulty of isolating pure jasmonic acid stereoisomers from both plants and fungi. It was reported that fungal cultures including *Diplodia sp.* produced mainly (+)-epijasmonic acid and a small amount of (-) jasmonic acid (12).

The methyl ester of jasmonic acid (methyl 3-oxo-2-(2'-pentenyl)-cyclopentaneacetate) is also known as both an active plant hormone and an important fragrance and flavor component with sweet-floral and jasmine-like aroma notes (13). Methyl jasmonate induces plant systems to provide defense from microbial infection, and is a secondary synthesis product of plants. Methyl jasmonate was also isolated from the Oriental fruit moth, *Grapholitha molesta* as a part of the male sex hormone (1,15). The authors demonstrated that the volatile form of jasmonic acid, methyl jasmonate, is a major signal molecule for inter- and intra species communications. Methyl jasmonate is expected to have

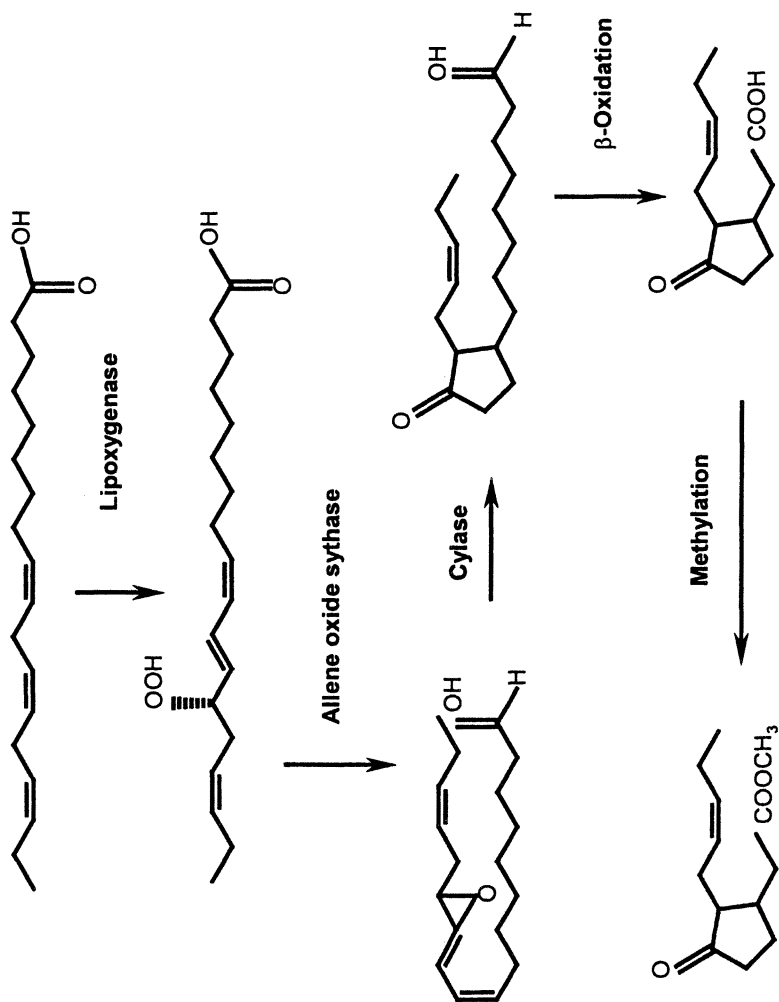


Figure 4. Metabolic pathway for the production of jasmonic acid and methyl jasmonate

potentially 4 optical isomers that may be formed (Figure 5). However, (1R,2S)-(+)-methyl epijasmonate was reported to be the biologically active form that contributed the characteristic odor in nature (14). Methyl epijasmonate was not easy to isolate at higher concentration due to the thermodynamically unstable nature of its stereospecific configuration.(4)

The schematic bioprocess to produce natural and kosher methyl jasmonate is summarized in Figure 6. The new commercial fermentation process was developed using *Diplodia sp.*, including *D. gossypina* ATCC 10936, that were isolated from citrus plants. *Diplodia sp.* had characteristic hyphal growth rather than pellet growth under submerged conditions (Figure 7). *Diplodia sp.* assimilated various carbon sources and exclusively synthesized (+)-jasmonic acid (>99%) in aerobic conditions as a part of de novo biosynthesis. The commercial fermentation process yielded >1.5 g/L of jasmonic acid in the fermentation broth after 15 days of fermentation using *D. gossypina* ATCC 10936. The resulting jasmonic acid was extracted from the fermentation broth using ethyl acetate. Crude jasmonic acid was recovered by stripping off the solvent at low temperature to prevent epimerization. The crude jasmonic acid was further purified by an anion exchange column method.

The esterification reaction of jasmonic acid with methyl alcohol was carried out in a high-pressure reactor to reduce epimerization. It was known that the cis-form of methyl jasmonate easily epimerized to the trans-form of methyl jasmonate under acid, base and high temperature conditions. The resulting methyl jasmonate mixture was recovered and purified by solvent extraction followed by fractional distillation. To improve the methyl epijasmonate concentration, additional steps of fractionation, such as the use of silica gel, were applied.

The final natural and kosher methyl jasmonate mixture contains various optical isomers, including >5% of biologically active methyl epijasmonate. The methyl jasmonate mixture can be used in creating powerful fresh and sweet impressions in flavor and fragrance formulations, and additionally can be used in secondary and tertiary flavor enhancement.

Summary

Recent consumer preference for “natural” flavors and fragrances prompted the flavor and fragrance industry to re-direct its attention to research in the production of flavor and fragrance ingredients via processes that would be considered “natural”. Especially, it is generally recognized in the industry that flavor and fragrance compounds having been prepared by microbial processes can be designated as natural products, and therefore, have an important place in

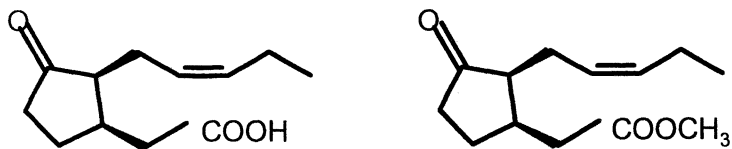


Figure 5. Configuration of biologically active (+) *epi*-jasmonic acid (3-oxo-2-(2-pentenyl)-cyclopentaneacetic acid) and (+) *epi*-methyl jasmonate (methyl 3-oxo-2-(2-pentenyl)-cyclopentaneacetate)

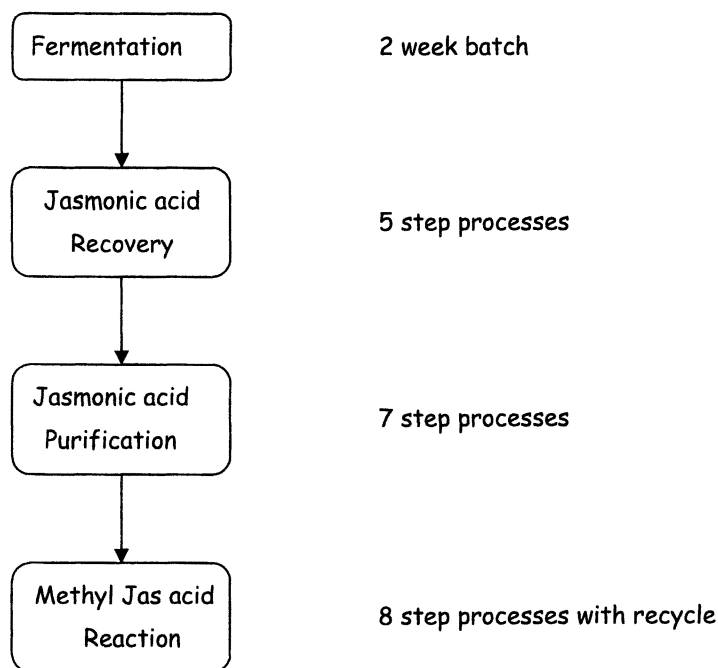


Figure 6. Flow diagram for the production of methyl jasmonate

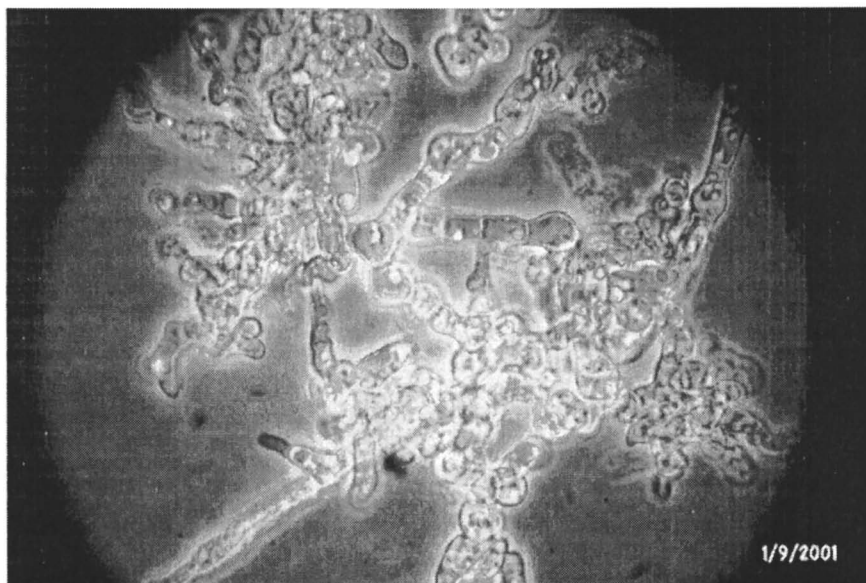


Figure 7. Diplodia gossypina ATCC 10936 with hyphal growth that is required for the production of jasmonic acid under submerged conditions

the commercialization of food and other consumer items. As the industry continues to use microbial metabolic properties for the production of natural flavor and fragrance chemicals, the new chemicals will become available for the creation of natural flavors and fragrances, providing important advantages in the market place. It is also noteworthy that another important global trend driving the flavor and fragrance industry is the growing interest in "label-friendly" ingredients reflecting health consciousness, convenience and environmental safety. Environmentally benign bio-organic syntheses should provide alternative ways to produce flavor and fragrance chemicals to meet consumer expectations. It is clear that research in flavors and fragrances should be multidisciplinary to meet the challenges and changes demanded by society and consumers. Recombinant DNA technology will be an important tool in the future, not only for the production of new flavor and fragrance chemicals, but also for improving future understanding of the mechanisms of olfaction and taste

References

1. Acree, T.E.; Nishida, R.; Fukami, H. *J. Agric. Food Chem.* **1985** 33, 425-427.
2. Adlercreutz, H. *Biotechnol. Letter* **1991** 13, 229-234
3. Creelman, R.A.; Mullet, J.E. *Annual Rev. of Plant Physiol. & Plant Molecular Biol.* **1997** 48, 355-381
4. Dart, R.K.; Kerry, S.; Marples, B.A. *Enzyme Microb. Technol.* **1992** 14, 954-958
5. Farbood, M.I.; Blocker, R.W.; Avizzigno, J.; Muralidhara, R.; US Patent 6,602,535, 2003
6. Farbood, M.I.; Kim, A.Y.; Blocker, R.W.; US Patent 6,482,794, 2002
7. Farbood, M.L.; Blocker, R.W.; McLean, L.B.; Sprecker, M.A.; McLean, M.P.; Kossiakoff, N.; Kim, A.Y.; Hagedorn, M.; US Patent 6,458,569, 2002
8. Farbood, M.; Morris, J.A.; Sprecker, M.A.; Bienkowski, L.J.; Miller, K.P.; Vock., M.H.; Hagedorn, M.L.; US Patent 5,110,953, 1992
9. Hilton, M.D.; Cain, W.J. *Applied Environ. Microbiol.* **1990** 56, 623-627
10. Hou, C.T.; Brown, W.; Labeda, D.P.; Abbott, T.P.; Weisleder, D. *J. Ind. Microbiol. Biotechnol.* **1997** 19, 34-38
11. Husain, A.; Ahmad, A.; Agrawal, P.K. *J. Natural Products* **1993** 56, 2008-2011
12. Miersch, O.; Schneider, G.; Sembdner, G. *Phytochemistry* **1991** 12, 4049-4051

13. Muheim, A.; Hausler, A.; Schilling, B.; Lerch, K. *Perfumer & Flavorist* **1998** *23*, 21-27
14. Nissida, R.; Acree, T.E.; Fukami, H. *Agric. Biol. Chem.* **1985** *49*: 769-772
15. Nishida, R.; Baker, T.C.; Roelofa, W.L. *J. Chem. Ecol.* **1982** *8*, 947-959
16. Schreiber, W.; Scharpf, L.G.; Katz, I. *Chemtech* **1997** March, 58-62
17. Simon; *Angew. Chem. Int. Ed. Engl.* **1985** *24*, 539-553
18. Staswick, P.E. *Plant Physiol.* **1992** *99*, 804-807
19. The Code of Federal Regulations 21 Food and Drugs, Parts 100-169
20. Tyrrell, M. *Perfumer & Flavorist* **1995** *20*, 13-21
21. Vick, B.A.; Zimmerman, D.C. *Plant Physiol.* **1984** *75*, 458-461
22. Wache, Y.; Aguedo, M.; Choquet, A.; Gatfield, I.L.; Nicaud, J-M.; Belin, J-M. *Applied Environ. Microbiol.* **2001** *67*, 5700-5704
23. Yang, W.; Dosal, L.; Rosazza, P.N. *Applied Environ. Microbiol.* **1993** *59*, 281-284

Chapter 5

Deuterium Distribution in Benzaldehyde Molecules by SNIF–NMR Analysis

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Deuterium site-specific distribution stores information about the history of a benzaldehyde product. Comparing deuterium site-specific distribution of an unknown sample with that of standard samples can identify the origin of an unknown sample. The precondition for successful identification is that the preparation procedures, such as isolation and purification, do not result in any deuterium abundance change or the changes are known and reproducible for a given process. In this paper, we report our findings on the deuterium shift during a manufacturing process and the effect on the SNIF–NMR results.

Introduction

With a characteristic aroma of bitter almond oil and a typical sweet cherry taste, benzaldehyde is widely used as an important ingredient for creating cherry, peach, vanilla, chocolate, and many other flavors. Benzaldehyde can be produced from botanical sources such as cinnamon, cassia, bitter almonds, pits of apricot, peaches, plums, and cherries, or from petroleum products such as toluene.

The market price of benzaldehyde varies widely, from more than \$400/kg to less than \$2/kg mainly based on its origin and production processes. Generally, benzaldehyde from bitter almond oil is the most expensive while benzaldehyde derived from cinnamic aldehyde is less costly and the synthetic products from toluene or benzyl chloride have the lowest price. Because of the price differences, it is crucial to establish a reliable analytical method to unambiguously distinguish benzaldehyde products of different origins. Analytical methods for determining product authentication have existed for decades, including trace analysis and isotope ratio analysis. SNIF-NMR (Site-specific Natural Isotope Fractionation by NMR), developed by Martin and co-workers (1,2,3), is probably the most widely accepted method, and based on the site-specific deuterium distribution on the benzaldehyde molecule. (SNIF-NMR is a registered trademark of Eurofins Scientific).

Deuterium has an average abundance of 0.015% of the hydrogen in the hydrosphere of the earth. However, it is not uniformly distributed on the earth. The isotope is twice as heavy as hydrogen, so compounds containing deuterium behave differently in some physical and chemical processes (isotope effects). The D/H ratio depends on the geographic location due to the water cycle of evaporation and condensation – an effect of naturally occurring fractional distillation and the centrifugal force of the earth's rotation. For example, the deuterium concentration of water is 90 ppm at the South Pole and 160 ppm at the equator. Because water is the only source of hydrogen for biosynthesis within a plant, the D/H ratio of a chemical compound formed in a plant depends on the geographic origin of that plant. The degree of isotope effect is determined by the chemical reaction mechanisms and the reaction dynamics. Therefore, the site-specific D/H ratio of a compound produced in the plant is also a function of the formation pathway, the climate, and the environment.

Deuterium site-specific distribution stores information about the history of a benzaldehyde product. Products from the same source should have similar isotope distribution. Therefore, comparing a test sample's deuterium site-specific distribution with that of standard samples can identify the origin of the test sample. The precondition for a successful identification is that the

preparation procedure, such as isolation and purification, does not result in any deuterium abundance change or that the changes are known and reproducible for a given process. Generally, these conditions are fulfilled in commonly-used manufacturing processes for benzaldehyde production. However, any unexpected deuterium shift will lead to a misidentification of the sample origin by SNIF-NMR analysis. In this paper, we report our findings on the deuterium shift during a manufacturing process and the effect on the SNIF-NMR results. A proposal is also presented to improve the data processing and interpretation of SNIF-NMR results.

Experimental

Sample preparation: Benzaldehyde (2.50 mL) was added to a 10mm constricted NMR tube. Tetramethylurea (TMU, 0.30 mL) was used as internal standard and hexafluorobenzene (0.05 mL) as locking solvent. The tube was flame sealed and the contents were mixed thoroughly before analysis.

NMR acquisition parameters: Deuterium NMR data (76.77 MHz) were recorded at 308°K using a Bruker DRX 500 spectrometer equipped with a 10mm SEX-2H probe with proton decoupling and fluorine lock (Hexafluorobenzene, Aldrich Chemical Co., Inc.), and a BCU-05 temperature controller. Four data sets were obtained from each sample using a 3.49 s acquisition time, 512 scans, 4 dummy scans, 10.88 microsecond 90° 2H pulse, and WALTZ16 decoupling. Each data set was processed with 1 Hz line broadening using automatic phasing and an integration macro.

Data processing parameters: The deuterium NMR peak area was normalized to peak area percentage. Principal component analysis (PCA) was conducted using SPSS software package. The final results were presented as a two dimensional PCA plot using the first two principal components.

Results and Discussion

Production Processes of Benzaldehyde

Benzaldehyde can be produced by hydrolysis of amygdalin, a glucoside present in the kernels of bitter almonds and in apricot pits. The formation of

benzaldehyde occurs when the crushed kernels or pits are boiled in water. This product is also called oil of bitter almond.

Retro-aldol hydrolysis of cinnamic aldehyde forms benzaldehyde and acetaldehyde. Interestingly, the reverse reaction, i.e., aldol condensation of benzaldehyde and acetaldehyde has also been used to produce cinnamic aldehyde. In the food industry, many users prefer benzaldehyde produced using cinnamic aldehyde isolated from cinnamon because it is a material with a natural origin and a reasonable price.

Benzaldehyde can also be synthesized by the oxidation of toluene or by the hydrolysis of benzyl chloride in the presence of an alkali.

Because benzaldehyde produced from different starting materials has an identical structure, it is a challenging task to differentiate these products. The presence of trace amounts of starting material and/or by-products in the final products provides information about the starting material or reaction pathways. Traditionally, trace analysis was performed using GC/MS for benzaldehyde authentication. It is often very difficult to get conclusive results using only GC/MS, especially when dealing with adulteration in a highly purified material or in blends containing a small amount of a synthetic product. The total deuterium content in benzaldehyde can also be used for authentication analysis. Natural benzaldehyde from bitter almond oil and from the retro-aldol reaction of cinnamic aldehyde has a deuterium concentration of 140 ppm. Synthetic benzaldehyde from hydrolysis of benzyl chloride has a deuterium concentration of 150 ppm, and from the oxidation of toluene, 230 ppm. Adjustments to the total isotope ratio of a sample can be easily accomplished by adding isotope-enriched or depleted material. The most definitive characterization of benzaldehyde from different origins is achieved by determining the distributions of deuterium at each molecular site. One can clearly differentiate the four most common types of benzaldehyde products based on knowledge of the distributions of deuterium at each molecular site.

Current NMR Methods for Authentication Analysis

The principle of SNIF-NMR is based on the fact that the natural biosynthesis of any organic compound generates a unique isotope distribution at different the molecular positions for that compound. These isotopic fingerprints are determined by the nature of the precursors, the chemical or biochemical synthesis mechanisms, the evolution of the environmental conditions, and even the physiological status of the precursor plant. Synthetic benzaldehyde has an almost perfectly random distribution of deuterium on the benzene ring, and

benzaldehyde from botanical material has certain enrichment/depletion deuterium on the molecular sites. Benzaldehyde from different botanical sources has different deuterium distributions due to different biosynthetic pathways.

Benzaldehyde obtained from bitter almond oil and from the retro-aldol reaction of cinnamic aldehyde from cinnamon originate from different biosynthetic pathways. Therefore, benzaldehyde from bitter almond oil can be easily differentiated from benzaldehyde ex-cassia by comparing their site-specific deuterium distributions.

As mentioned above, benzaldehyde can be synthesized from toluene or benzylchloride. Benzylchloride is normally produced by the chlorination of toluene. Therefore, the synthetic product, benzaldehyde, has the same starting material, toluene, which is commonly produced from paraffins in a refinery process. It has been observed that there is a significant difference in the relative molar fraction of deuterium on the formyl site of benzaldehyde between the products from oxidation of toluene (56%) and from hydrolysis of benzylchloride (18%). This is due to the isotope effects during the oxidation of toluene: the elimination of the lighter hydrogen atom from the methyl group is preferred over elimination of the deuterium atom so that the remaining deuterium in the aldehyde group is enriched.

While the deuterium spectra of benzaldehydes from different sources do not appear (upon visual inspection) to have significant differences from one another, other than some variation in intensity, the deuterium distribution of benzaldehyde does form clusters on a principal component analysis plot. Benzaldehyde products from the same source have similar deuterium distributions and are therefore close to each other on the plot (Figure 1). Thus, the origin of benzaldehyde can be differentiated based on site-specific deuterium distribution. Products outside of the clusters of known samples are normally considered as originating from an unknown source or as a mixture of benzaldehyde from different known sources.

Abnormal Deuterium Distribution

Interpretation of the SNIF-NMR results is based on knowledge of existing products and manufacturing. The commonly used physical processes, e.g., isolation and purification, do not alter the isotope ratio of benzaldehyde due to the close similarity of properties among the mono-deuterated and non-deuterated benzaldehydes so that the final product conserves the original state

of isotope distribution of the molecules. The retro-aldol reaction of cinnamic aldehyde has no significant isotope effects on the final product. So benzaldehyde formed by the reaction retains the site-specific deuterium distribution of cinnamic aldehyde.

When the distribution pattern in deuterium of a product does not match that of any known product, one would consider one of two causes: a) adulteration, i.e., mixed products from different sources; b) the product is from an unknown source.

However, abnormal deuterium distribution does not always result from adulteration or unknown sources. It is known that auto-oxidation of benzaldehyde will enrich the deuterium distribution on the formyl site of the remaining benzaldehyde (4,5). Therefore, old samples may have a higher molar fraction of deuterium at position 1. This same reaction does not affect the isotope distribution on the aromatic moiety.

Incomplete recovery from a chromatographic separation may also produce isotopic fractionation. It has been reported that physical processes, such as chromatographic separation, applied to vanillin can lead to isotopic fractionation (separation of heavy molecules from the light molecules) (6). Such phenomena may slightly change the values obtained from the isotopic measurements and, therefore, if adequate care is not taken, could lead to erroneous interpretation of the results (7).

Recently, we also observed abnormal deuterium distribution of a sample of benzaldehyde, which deviated significantly from the expected cluster formed by benzaldehydes produced from cinnamic aldehyde. Adulteration and use of a starting material from an unknown source were ruled out. Experiment results indicated that the deuterium shift was not due to oxidation and isotope exchange. No chromatography process was used. Our attention focused on the distillation process.

To purify the crude product after retro-aldol reaction of cinnamic aldehyde, distillation was performed in three stages: the first stage was to remove the highly volatile impurities under a high reflux rate, and a small fraction of pure benzaldehyde was also collected. The second stage was to obtain the main fraction of benzaldehyde. The third stage was to collect the remaining benzaldehyde under a low reflux rate. Aliquot samples were collected during a distillation process, and the site-specific deuterium distribution was determined by NMR.

As shown in Figure 2, the deuterium abundance on the carbonyl site has a slight but significant increase as the distillation proceeded while the deuterium concentration on all sites of the aromatic moiety remained practically constant

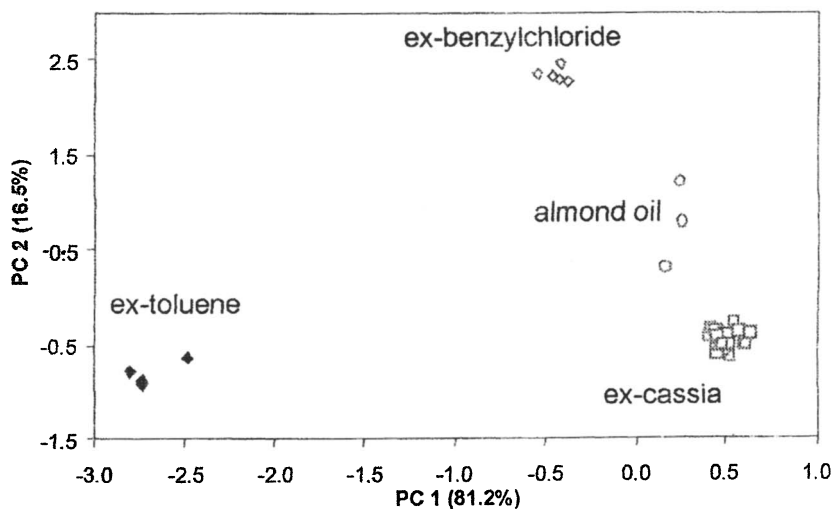


Figure 1. PCA plot of benzaldehyde products based on deuterium distribution.

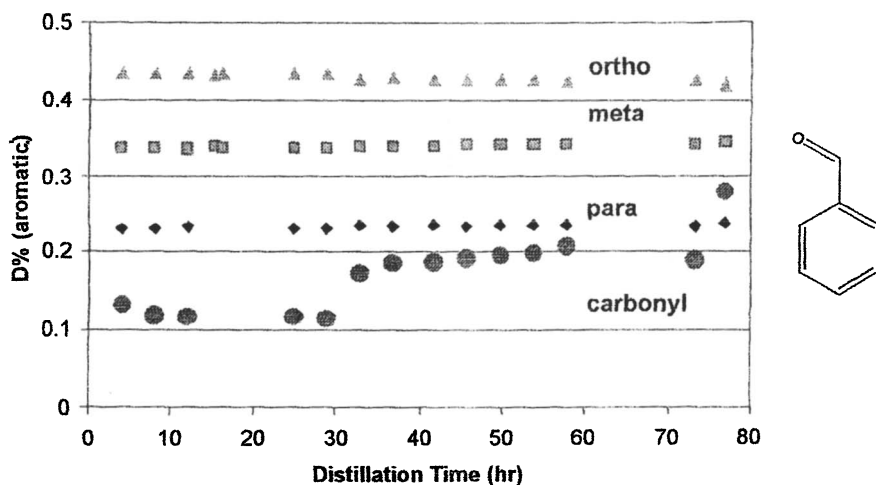


Figure 2. Site-specific deuterium concentration of distillation fractions.

during the whole distillation process. More details on the deuterium changes on the carbonyl site are presented in Figure 3. The lowest concentration of deuterium was found in the distillate from the first stage of the distillation when the highest reflux rate was set, and the distillate was composed of predominantly the most volatile components. As expected, the distillate from the last stage of the distillation has the heaviest components. It is concluded that on the carbonyl site, the light distillate has a depleted deuterium concentration, and heavy distillate is enriched in deuterium. The deuterium content of the carbonyl site of the main fraction from the second stage of distillation represented the average isotope distribution of the product.

This fractional distillation effect on deuterium concentration is also shown clearly on the PCA plot of the deuterium distribution (Figure 4). Both light and heavy distillates are located outside of the normal distribution cluster. The light distillate from the first stage of distillation has the highest score on the first principle component, which has the highest loading of carbonyl site deuterium. The heaviest distillate from the last stage of distillation is located opposite of the light fraction, while the main fraction fell into the normal distribution cluster of benzaldehyde from cinnamic aldehyde.

Although the deuterium concentration shifts due to the distillation process did not change the origin of the compound, the current SNIF-NMR method will deliver a false negative result in such cases. In order to avoid product rejection based on false negative results of SNIF-NMR results, manufacturers should use only the main fraction as the final product and discard the first and the last fraction during the distillation. If higher yield is desirable, all fractions from the distillation should be mixed together to ensure a complete recovery.

As discussed above, synthetic benzaldehyde from toluene has enriched deuterium on the carbonyl site, so false positive results can be obtained when the light distillate is combined with a synthetic benzaldehyde in the right ratio. Therefore, using the SNIF-NMR results as the only criteria for authentication approval would not be recommended. We conducted a preliminary study to explore the possibilities to improve the current SNIF-NMR method.

Improvement of the SNIF-NMR Method

The carbonyl group of benzaldehyde is the compound's most active site for many reactions. The deuterium concentration on this site is prone to change due to isotope effects. During the oxidation of toluene, deuterium enriches at the carbonyl site while deuterium abundance on the aromatic moiety remains practically unaffected. Apparently, the mono-deuterated benzaldehydes have

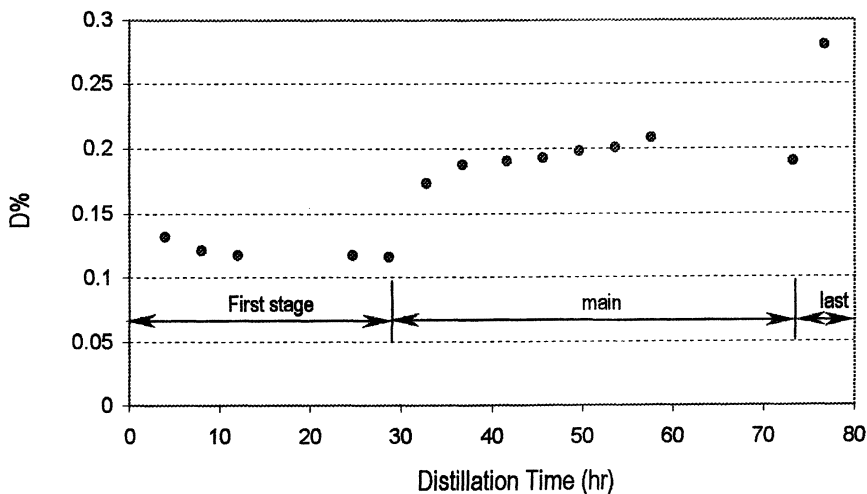


Figure 3. Relative deuterium concentration on the carbonyl site of benzaldehyde

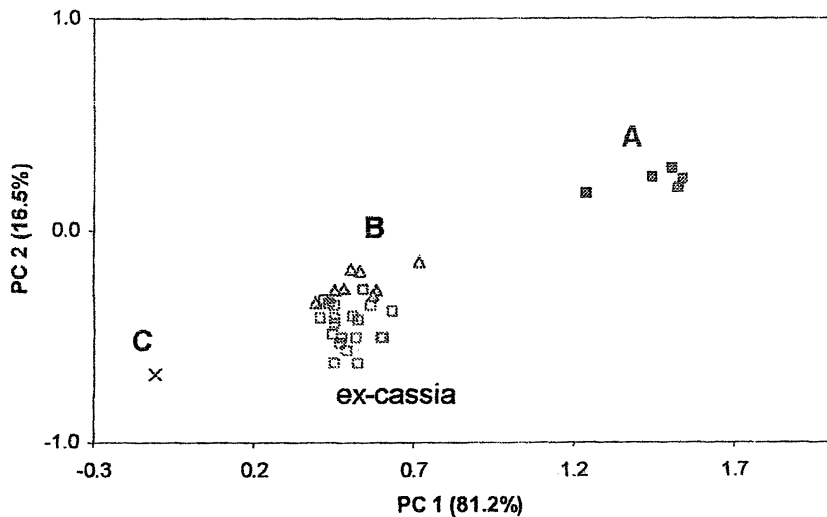


Figure 4. PCA plot of abnormal deuterium distribution of benzaldehyde products

A: First Distillate, B: Main fraction, C: Last distillate.

different physical properties. Benzaldehyde deuterated on the carbonyl site is clearly more different than the benzaldehyde deuterated on the aromatic moiety, as it can be seen from the deuterium shift during the distillation process. Therefore, deuterium distribution on the carbonyl site is also vulnerable to be changed by a physical process.

The deuterium atoms on the aromatic sites are more stable and show a consistency in concentration. So far, no significant changes in the aromatic deuterium concentration have been observed after the manufacturing processes discussed above. The aromatic deuterium distribution also forms a “finger print” regarding the origin of benzaldehyde: the aromatic moiety of benzaldehyde from a fossil source has a random deuterium distribution. The aromatic deuterium distribution is very characteristic for benzaldehyde from botanical sources. Figure 1 shows the PCA plot based on the deuterium site-specific molar fractions on the aromatic moiety of the benzaldehyde samples. As expected, the clusters of benzaldehyde ex-toluene and ex-benzylchloride in Figure 1 are now merged together in Figure 5 because deuterium has the same statistical distribution on the benzene ring of the benzaldehyde products from the fossil origin. Benzaldehyde from almond oil is clearly different from benzaldehyde ex-cassia. Note that the X and Y scales differ in Figures, 1, 4 and 5, because the plots are based on different data sets. The relative location of the data points and their separation into clusters is more significant than their relationships to the numbers on the scales.

Because the deuterium distribution on the aromatic sites reserves the original state of the starting material, while the carbonyl deuterium is labile during the production, only the aromatic deuterium abundance should be used as the primary data for the discrimination analysis of benzaldehyde products.

Conclusions

SNIF-NMR is a powerful method for the authentication analysis of benzaldehyde products. However, manufacturing processes may cause the deuterium to shift on the carbonyl site. In some cases, the current SNIF NMR method cannot differentiate abnormal distribution of deuterium caused by adulteration or by production processes that lead to false negative or false positive conclusions of a product. Deuterium on the aromatic sites of benzaldehyde is more stable and is not affected by the known manufacturing processes. The aromatic deuterium distribution is very specific and can be used to classify benzaldehyde products from fossil or different botanical origins. The proposed improvement needs to be further developed and validated.

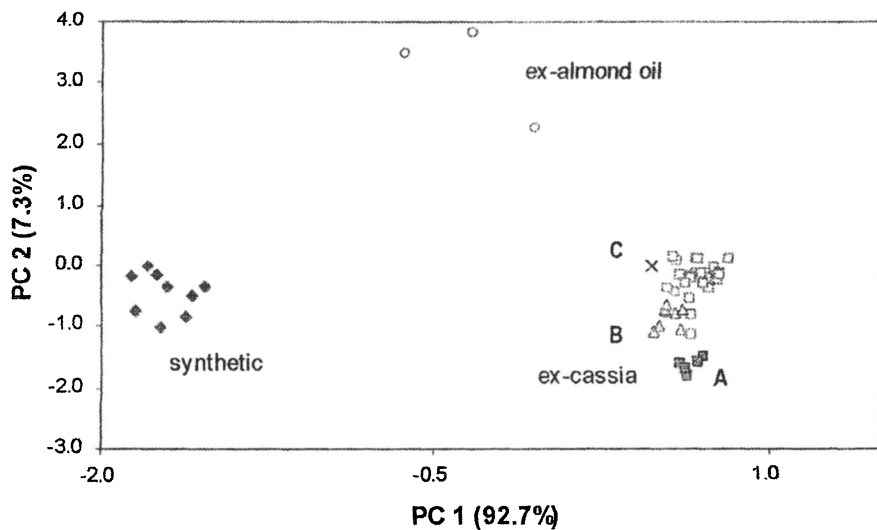


Figure 5. PCA plot of benzaldehyde samples based on aromatic deuterium distribution.

A: First Distillate, B: Main fraction, C: Last distillate.

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References

1. Martin, G. J.; Martin, M. L. Deuterium labeling at the natural abundance level as studied by high field quantitative ²H NMR. *Tetrahedron Lett.*, **1981**, *22*, (36), 3525-3528.
2. Hagedorn, M. L. Differentiation of Natural and Synthetic Benzaldehydes by ²H Nuclear Magnetic Resonance, *J. Agric. Food Chem.*, **1992**, *40*, 634-637.
3. Remaud, G.; Debon, A. A.; Martin, Y.; Martin, G. G.; Martin, G. J. Authentication of Bitter Almond Oil and Cinnamon Oil: Application of the SNIF-NMR Method to Benzaldehyde, *J. Agric. Food Chem.*, **1997**, *45*, 4042-4048.
4. M. L. Hagedorn, Differentiation of Natural and Synthetic Benzaldehydes by ²H Nuclear Magnetic Resonance, *J. Agric. Food Chem.*, **1992**, *40*, 634-637.
5. M. Butzenlechner, A. Rossmann, and H.-L. Schmidt, Assignment of Bitter Almond Oil to Natural and Synthetic Sources by Stable Isotope Ratio Analysis, *J. Agric. Food Chem.*, **1989**, *37*, 410-412.
6. Hoffman, P.; Salb, M. Isolation and stable isotope ratio analysis of vanillin. *J. Agric. Food Chem.* **1979**, *27*, 352- 355.
7. Fellous, R.; George, G.; Schippa, C. Vanilline : Anomalies dans le protocole d'identification par ¹³C. (Vanillin: nomalies in the carbon-13 identification protocol.) *Parfums, Cosme't., Aro'mes.* **1992**, *106*, 95-98.

Chapter 6

Analysis of Flavors Using a Mass Spectral-Based Chemical Sensor

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Abstract

A mass spectrometer based chemical sensor was used to model citrus peel oils. Seven samples and one duplicate were analyzed and subsequently modeled with multivariate analyses such as principal component analysis (PCA) and Soft Independent Modeling of Class Analogy (SIMCA). Discrimination between samples was excellent with clear distinctions between the samples and considerable overlap of the duplicate sample. Samples were also analyzed with a gas chromatograph/mass spectrometer (GC/MS) using similar multivariate models. GCMS results were similar to the ones obtained by sampling the static headspace, but with longer analysis times. Statistics obtained using SIMCA analysis allowed for the determination of which samples were most similar to a “target” sample.

Introduction

Citrus peel oils are generally collected from fruit as it processed into juice. These citrus oils are used as flavorings in numerous products from juices to cosmetics. There are many factors that affect the aroma of the peel oil, such as type of fruit, harvest maturity, processing affects, storage, and others (1, 2, 3, 4).

Traditional analysis of citrus peel oils involves the use of gas chromatography (GC) utilizing a mass spectrometer (MS) as a detector and quantitating various chemical compounds as “markers” (5, 6, 7, 8, 9). Although, GC/MS is a reliable technique, the analysis times can be long (30-60 minutes) and its output (a total ion chromatogram, TIC) can be difficult to compare when many samples are analyzed. A slightly different approach is to utilize a mass spectrometer (MS) as an electronic nose/chemical sensor. In the case of the MS, each mass to charge (m/z) is used as a ‘sensor’. There is no chemical separation of the sample prior to analysis which means that the mass spectra collected are of the entire product. This is slightly different from the traditional use of a MS—where chemically pure compounds are introduced into the MS as they elute from a GC. This lack of chemical separation is similar to the electronic nose’s method of introducing the sample to the sensor array without chemical separation. The chemical sensor used in the static headspace mode provides high sample throughput. The autosampler is directly coupled to a mass spectrometer without a GC which results in MS analysis times of 1-3 min/sample. Multivariate data analysis is then applied to the samples’ mass spectral fingerprints and comparison of samples profiles can be easily done with exploratory data analysis (10). The different methods, electronic nose, GC, and MS-based chemical sensor have been compared (11).

Several multivariate techniques are useful in analyzing the chemical sensor data. Principal component analysis (PCA) is a multivariate technique that reduces the dimensionality of the data sets by building a new set of coordinates, principal components or PCs. These PCs are linear combinations of the original variables and they are orthogonal to each other and therefore uncorrelated. They are also built in such a way that each one successively account for the maximum variability of the data set (12). This is useful for visualizing the data to determine if there is inherent structure.

Classification of the citrus oils was possible by using Soft Independent Modeling of Class Analogy (SIMCA). This type of algorithm is designed to compare new samples against previously-analyzed sets. Another ability if SIMCA is the determination if a sample does not belong to any predefined class.

The combination of a MS based chemical sensor has many possible applications. The most common application is with quality assurance/quality control (QA/QC) (13, 14, 15). A multivariate model is generated according to product type and when a sample deviates from that model, it indicates a

problem. A second application of this type of multivariate modeling is adulteration (16, 17). The method is similar to the QA/QC application in that sample that fall outside the model are suspect of adulteration.

The same data analysis that is applied to the MS based chemical sensor data can also be used to examine traditional GC/MS data. This is accomplished by using special macros that create the samples' mass fingerprints by adding the all the scans present in the total ion chromatogram (TIC). The resulting two-dimensional data set can be analyzed with multivariate software.

Experimental

Eight different lime and lemon flavor formulations were provided by a commercial flavor company (Table I). Six replicas of each flavor were analyzed using 7.5 μ L aliquots. The aliquots were placed in 10 mL vials which were crimped and equilibrated for 15 minutes at 60 °C before static headspace sampling. The headspace parameters were: 15 min incubation, 65 °C syringe, 0.75 min flushing of syringe after injection, cycle time of 4 min. Two mL were filled and injected at a 250 μ L/s . There is no column for a separation prior to the mass selective detector (MSD), the entire headspace of each sample is introduced into the MSD.

Table 1. Description of lemon and lime samples.

Sample Number	Type	Description ¹
1	Lemon	FTNF
2	Lime (Persian)	FTNF
3	Lemon	FTNF
4	Lemon	FTNF (same as #1)
5	Lemon	Natural
6	Lemon	WONF
7	Lemon-Lime	WONF
8	Lime (Key)	Natural

¹FTNF=From The Named Fruit, WONF=With Other Natural Flavors

Natural and WONF flavors are not solely from named fruit and may have other flavors added or solvents

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The mass spectrum of each of the eight flavor samples was acquired in 1.20 minute runs in the scan mode using a Gerstel ChemSensor. The carbon dioxide peak (from the air in the sample) and the ethanol peaks were avoided by scanning from 48 m/z to 160 m/z range using 70eV of ionization energy.

All the eight flavor samples were also analyzed using a 6890 GC coupled to a 5973 MSD, both from Agilent Technologies. This instrument was equipped with a thermal desorption autosampler. The samples were diluted 10,000 fold and extracted for one hour with 'Twister' stir bar sorptive extraction; (SBSE). Both the thermal desorption autosampler and the 'Twister' are from Gerstel. The autosampler was operated in the standard mode, splitless. The transfer temperature was 275 °C with an initial temp of 20 °C held for 0.4 min and then ramped at 60 °C/min to 250 °C and held for 5 min. The volatiles from the SBSE were then trapped on a CIS 4 operating in the split mode. The initial temp was – 120 °C held for 0.2 min and then ramped at 12 °C/min to 280 °C which was held for 3 min. The GC oven was initially at 40 °C for 2 min and then ramped to 280 °C at 10 °C/min and held for 5 min. The MSD was scanned from 35 to 350 m/z with a 70 eV ionization energy.

The two data matrices, one for the static HS and another one for SBSE (18, 19), were created using ChemStation DA with Gerstel macros. For multivariate data analysis Pirouette 3.11 and InStep 2.11 were used. Both data sets were mean-centered and normalized before creating the PCA and SIMCA models. Statistica (Statsoft) v6 was used for ANOVA.

Results and Discussion

Static Headspace analysis

Using the mass spectrometry based chemsensor the entire headspace volatiles of each of the 8 flavor samples were sampled and a characteristic fingerprint mass spectrum was obtained. For example, Figures 1 and 2 represent the TIC and MS obtained for formulations #1 and #8 respectively. Inspection of these two figures indicates that each flavor sample has a characteristic mass spectrum. Similar results were observed with the mass spectrum from the rest of the flavors formulations. Even though the mass spectral fingerprints of the citrus oils were similar there were subtle differences in some ion ratios.

Figure 3 contains the mass spectra line plot of all 8 formulations used for developing the chemometric models. These lineplots contain the mass spectral information in which a line connects the ions abundances. A closer look of the data is seen in the inset that shows how the different flavor samples have different ion abundance and therefore different mass spectral fingerprints. These

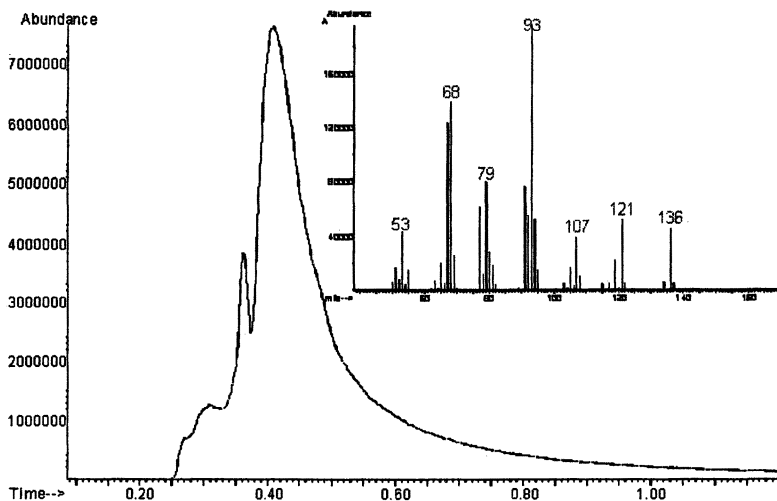


Figure 1. TIC and MS of flavor #1 obtained with headspace chemical sensor.

results also indicate excellent sample reproducibility since each band represents 6 replicas for each sample.

The quality of the data collected was checked using exploratory data analysis. Principal component analysis (PCA) is a type of algorithm that is consider exploratory because it provides the best view of variability without assigning specific categories to each type of class. Exploratory analysis also indicates if the data is appropriate for building a classification model. PCA searches for correlations among all m/z abundances simultaneously and extracts linear combinations of highly correlated m/z abundances (principal components, or PCs) that describe the greatest amount of sample variability. In this particular data set, 95 % of the variance was captured within the first three PCs.

The PCA scores plot is a scatter plot of the samples projections into the new coordinates. A PCA scores plot is shown in Figure 4, in this plot close distance between points indicates close similarities in the samples mass spectra. For example, formulas #1, #4, #3 and #6 cluster close together which suggests similar chemical composition. On the other hand, samples far away from others such as formula #2 indicate very unique composition. A close examination of

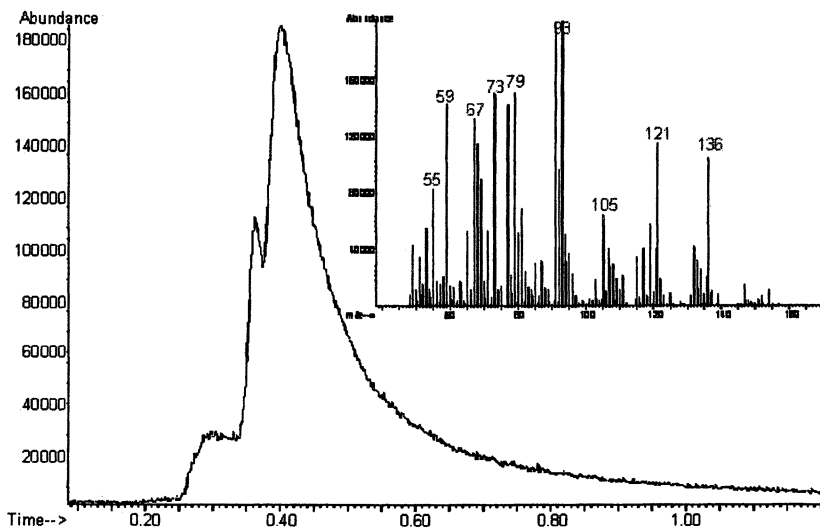


Figure 2. TIC and MS of flavor #8 obtained with headspace chemical sensor

the mass spectrum of this sample indicates differences in ion abundances compared to the other samples. Inspection of Figure 4 also indicates very little differences between formulas #1 and #4 since they cluster together. Overall the samples projections into the PCA model follow closely the type of flavor as seen in Table I.

The quality of the data collected was checked using exploratory data analysis. Principal component analysis (PCA) is a type of algorithm that is consider exploratory because it provides the best view of variability without assigning specific categories to each type of class. Exploratory analysis also indicates if the data is appropriate for building a classification model. PCA searches for correlations among all m/z abundances simultaneously and extracts linear combinations of highly correlated m/z abundances (principal components, or PCs) that describe the greatest amount of sample variability. In this particular data set, 95 % of the variance was captured within the first three PCs.

The PCA scores plot is a scatter plot of the samples projections into the new coordinates. A PCA scores plot is shown in Figure 4, in this plot close distance between points indicates close similarities in the samples mass spectra. For

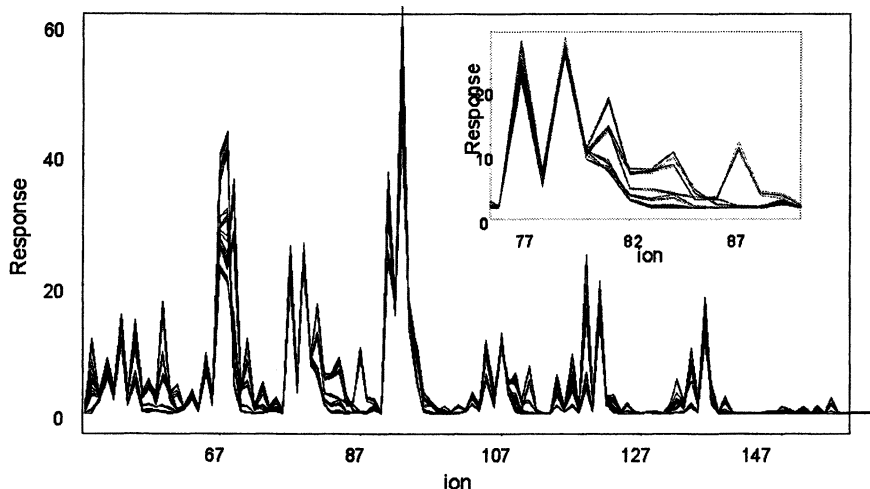


Figure 3. Normalized MS data for the eight different flavor samples.

example, formulas #1, #4, #3 and #6 cluster close together which suggests similar chemical composition. On the other hand, samples far away from others such as formula #2 indicate very unique composition. A close examination of the mass spectrum of this sample indicates differences in ion abundances compared to the other samples. Inspection of Figure 4 also indicates very little differences between formulas #1 and #4 since they cluster together. Overall the samples projections into the PCA model follow closely the type of flavor as seen in Table I.

A multivariate classification model was created with the above data. Soft-independent-modeling-class-analogy (SIMCA) uses PCA to model the shape and position of the samples. An acceptance region is then created for each different type of class. SIMCA models also provide interclass distances between samples, these distances are reported on Table II.

Table II. SIMCA-Interclass distances between samples when static headspace was collected

	#1	#3	#4	#6
#1	0.0	24.6	6.4	7.2
#3	24.6	0.0	34.2	24.3
#4	6.4	34.2	0.0	11.3
#6	7.2	24.3	11.3	0.0

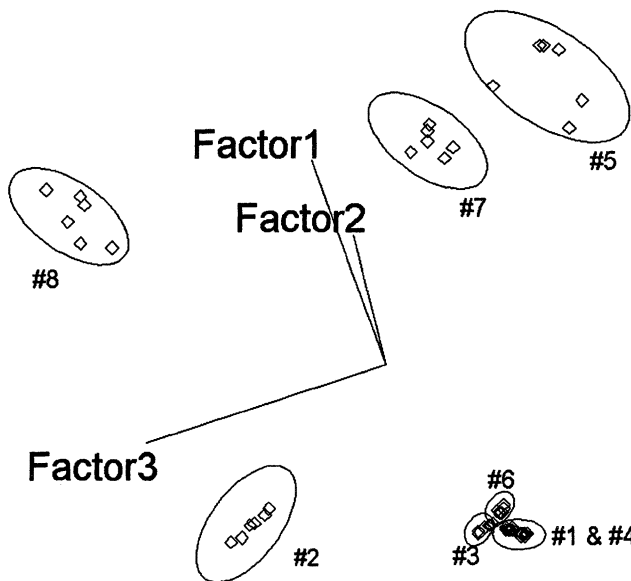


Figure 4. Projection of flavor samples into the space of the first three principal components with data acquired using the headspace chemical sensor.

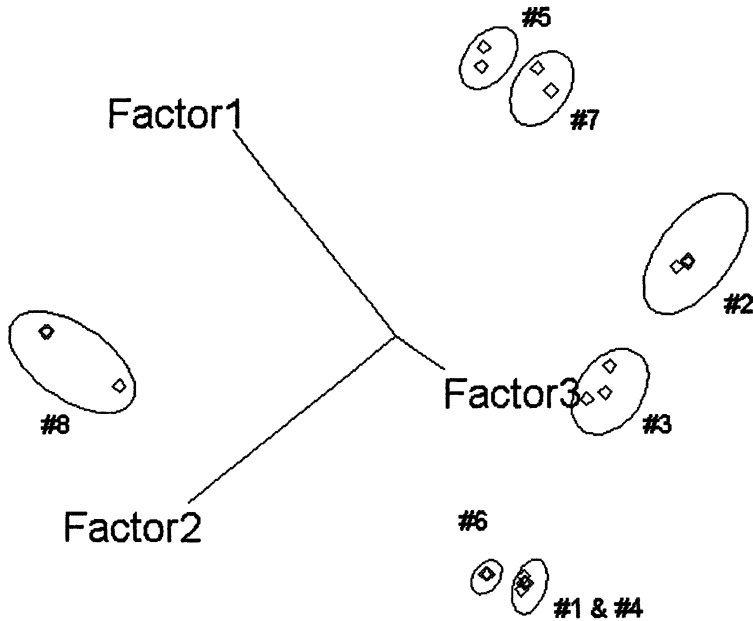


Figure 5. Projection of flavor samples into the space of the first three principal components with data acquired using stir bar sorptive extraction and GC/MS.

The larger the interclass distance between two groups, the better separated the groups. As a rule of thumb, interclass distance of 3 or greater indicates good separation between the samples. Interclass distance of less than 3 indicates small differences between the samples. For this analysis, it can be seen that the smallest interclass distance is between flavors # 1 and # 4 with a value of only 6.4. This small interclass distance between samples # 1 and # 4 also indicates similar mass spectral fingerprints and therefore similar chemical composition when the headspace is sampled.

Results after analysis of variance (ANOVA) are displayed in Table III. In this table each sample is compared with the others and the number of m/z's that are statistically different are listed. It can be seen that flavor #1 and flavor #4 only have 4 m/z that were different. Inspection of the masses that were found statistically different between samples #1 and # 4 revealed masses with low abundance (ions 60, 120, 134 and 135). This shows clearly that flavor #1 is quite similar to flavor #4 and also flavors #3 and #4 are similar as well.

Table III. Number of m/z that ARE statistically different between samples when static headspace was collected.

Flavor #	1	2	3	4	5	6	7	8
1	0							
2	82	0						
3	13	82	0					
4	4	86	11	0				
5	78	101	81	80	0			
6	81	97	96	83	78	0		
7	90	101	86	93	90	83	0	
8	93	103	96	94	72	86	88	0

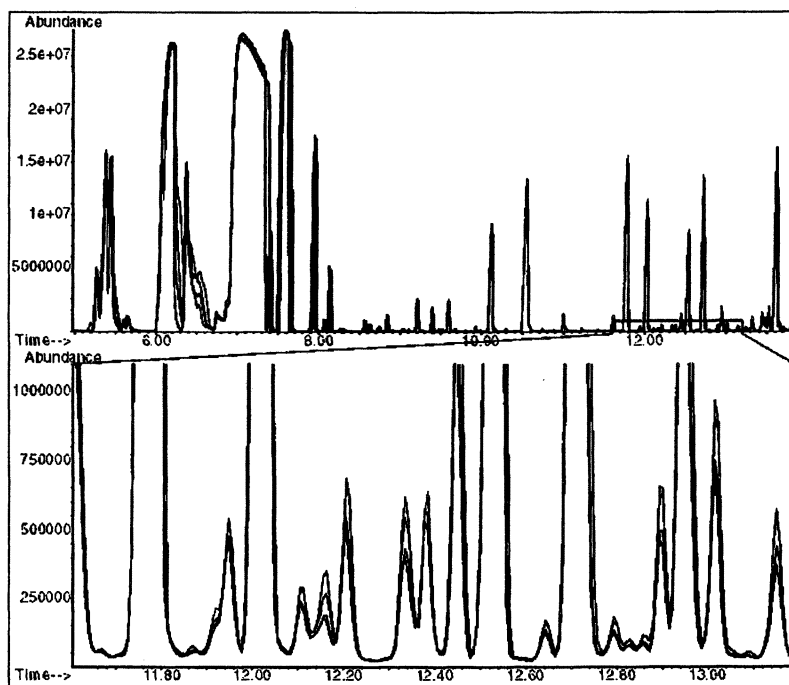
Stir Bar Sorptive Extraction (SBSE)

SBSE was carried out using stir bars that consist of a magnetic stir bar enclosed in glass coated with a polydimethylsiloxane (PDMS) phase. All flavors were analyzed using SBSE extraction followed by thermal desorption-GC/MS. The resulting chromatograms for the eight samples indicated some differences between them. This GC/MS data was reprocessed using special macros and characteristic fingerprints were obtained for each oil. These macros create an

ASCII file that contains a table of ions and their abundance for each sample in the sequence.

A PCA scores plot for the GC/MS flavors is shown in Figure 5. It can be seen in this figure that the clustering is similar to the one obtained with static HS, for example formulas #1 and #4 cluster very tight, which can also be seen in Figure 4. Figure 6 contains a TIC overlay of formulas #1 and #4, both in replicas. It can be seen from Figure 6 that the chromatograms for formulas #1 and #4 match very closely. These results are in agreement with the results obtained with the headspace chemical sensor.

The biggest advantage of using the chemical sensor instead of SBSE GC/MS analysis lies in the time savings. For example, the analysis of 50 samples required 4 hours using the headspace chemical sensor, while a single SBSE GC/MS run was 30 minutes.



*Figure 6. Overlay chromatograms of flavor #1 and flavor #4 acquired using SBSE and GC/MS. Lower trace is an expansion of a portion of the upper trace. (Reproduced with permission from *The Application Notebook February 2004*, page 40. Copyright 2004.)*

Conclusions

Differences between different formulations were detected using a mass spectral based chemical sensor with static headspace introduction. Results were validated using traditional GC/MS with SBSE-thermal desorption introduction. Advantages of using the chemical sensor include fast sample throughput and easy to interpret results. For this particular application, flavor formula # 1 was found to be very similar to flavor formula # 4. This result was also confirmed by the flavor supplier.

References

1. Shaw, P.E. Citrus essential oils. *Perfumer & Flavorist*, **1979**, 3(6), 35-40.
2. Verzera, A.; Russo, C.; La Rosa, G.; Bonaccorsi, I.; Cotroneo, A. *J. Essent. Oil Res.*, **2001**, 13(5), 343-347.
3. Usai, M.; Picci, V.; Arras, G. *J. Essent. Oil Res.*, **1996**, 8(2), 149-158.
4. Sawamura, M.; Shichiri, K.I.; Ootani, Y.; Zheng, X.H. *Ag. Bio. Chem.*, **1991**, 55(10), 2571-2578.
5. Chamblee, T.S.; Clark Jr., B.C. *J. Essent. Oil Res.*, **1997**, 9(3), 267-274.
6. Pino, J.A.; Acervedo, A.; Rabelo, J.; Gonzalez, C.; Escandon. *J. Essent. Oil Res.* **1999**, 11(1), 75-76.
7. Schieberle, P.; Grosch, W. *Leben. Wiss. Und Tech.*, **1988**, 21(3), 158-162.
8. Tamura, H.; Yang, R.H.; Sugisawa, H. Aroma profiles of peel oils of acid citrus. ACS Sym. Series #525, American Chemical Soc., 1993, 121-136.
9. Mondello, L.; Verzera, A.; Previti, P. Crispo, F.; Dugo, G. *J. Ag. Food Chem.*, **1998**, 46(10), 4275-4282.
10. Marcos Lorenzo, I. et al. *Anal. Bioanal. Chem.* **2002**, 374, 1205-1211.
11. Goodner, K.L.; Margaría, C.A., Kinton, V.R.; Baldwin, E.A. *Proc. Fla. State Hort. Soc.* **2002**, 115:46-49..
12. Massart, D. L.; Vandeginste, B. G. M.; Deming, S. N.; Michotte, Y.; Kaufman, L. *Chemometrics: A textbook*; Elsevier: Amsterdam, 1988; V. 2.
13. Steuer, B.; Schulz, H.; Lager, E. *Food. Chem.* 2001, 72(1), 113-117.
14. Cohen, E. *Zeitschrift-fuer-Lebensmittel-Untersuchung-und-Forschung*, **1983**, 177 (6), 468-471
15. Shaw, P.E. *Food Process.*, **1993**, 54(11), 103-104.
16. Mosandl, A.; Juchelda, D. *J. Essent. Oil Res.*, **1997**, 9(1), 5-12.
17. Vaira, S.; Mantovani, V.E.; Robles, J.C; Sanchis, J.C.; Goicoechea, H.C.. *Analytical Letters*, **1999**, 32(15), 3131-3141.
18. Tienpont. B., David, F., Bicchi, D., Sandra, P. *J. Microcolumn Separations*, **2000**, 12(11) 577-584
19. Baltussen, E., Sandra, P., David, F., Cramers, Carel *J. Microcolumn Separations*, **1999**, 11(10) 737-747

Chapter 7

Isotope Ratio Analysis by HRGC-MS of Monoterpene Hydrocarbons from Citrus Essential Oils

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The isotope ratio of monoterpene hydrocarbons in citrus essential oils of different origins was measured by ordinary high-resolution gas chromatography-mass spectrometry (HRGC-MS). The isotope ratio (Ir) was determined by the ratio of the isotope peak intensity (m/z 137) to the molecular mass peak intensity (m/z 136) of the monoterpene hydrocarbons. The accuracy of the Ir was examined by measuring monoterpene hydrocarbon standards and ¹³C-labeled compounds. The isotope fingerprints based on the values of monoterpene hydrocarbons from lemon, lime and yuzu essential oils were determined. These citrus essential oils were also discriminated by a principal component analysis of their Ir data. The characteristic vectors showed that α -terpinene, β -pinene and β -phellandrene were important components for distinguishing between the citrus species. It is suggested that this technique will be applicable to evaluate the quality, genuineness and origin of citrus fruits and their products.

Introduction

The natural abundance of the isotopes of each element is distributed in a given ratio. Plants on the Earth first convert solar energy into biochemical energy; the food chain starts from plants. Higher plants fix CO₂ by the Calvin-Benson cycle to biosynthesize various organic compounds for their constituents. (1) It is known that the enzyme, ribulose-1, 5-diphosphate carboxylase, differentiates a small mass difference between ¹²CO₂ and ¹³CO₂ when it fixes CO₂ in the atmosphere. This function is the so-called "isotope effect". It has also been stated that this effect could be achieved by every kind of enzyme involved in biosynthetic and metabolic pathways. (2) Thus, the isotope effect should also occur in the essential oils comprising terpene compounds. Every species, variety or strain of plant has substantially individual characteristics. Even among the same cultivar, different growing conditions such as the annual atmosphere and moisture, soil and fertilizers bring about small but appreciable differences in composition.

Citrus fruits are widely cultivated between the tropical and temperate zones in both the northern and southern hemispheres. The citrus fruit is one of the most important commercial crops, since it provides us with a pleasant taste, flavor and fragrance. It is said that there are thousands of varieties of citrus in the world. The extensive research on citrus flavor has been reviewed. (3,4) It has recently become commercially important to evaluate the property, quality, origin and genuineness of raw and processed products. Flavor analysis is a good means of revealing the characteristics of products, although it may be difficult to make sufficient discrimination among the same or similar cultivars by means of a general flavor compositional analysis by GC and GC-MS.

Faulharber *et al.* (5,6) and other researchers (7-9) have described how the determination of the isotope values of constituents is of increasing importance, especially in view of the demand for authenticity control and origin determination of essential oils and foods. To determine isotope values, gas chromatography-isotope ratio mass spectrometry (GC-IRMS) has been used, although not widely. The present authors (10) have studied the possibility of a more convenient and common means of analysis of isotope values, based on the isotope peak in the mass spectrum of a compound. The present study focuses on the development of a new analytical method for the differentiation of quality in commercial citrus oils of various origins.

Material and Methods

Materials

The samples of lemon and lime essential oils were commercial products for flavor materials. Yuzu fruits were collected from 10 local wholesale markets from northern to southern Japan in November 1999, and their cold-pressed oils (CPO) were prepared as described in a previous paper. (10) Lemon CPO was prepared from commercially sold fruits by the same method. Authentic chemicals for mass spectrometry were obtained from the commercial sources mentioned in a previous paper. (11)

Gas Chromatography-Mass Spectrometry (GC-MS)

Gas chromatography combined with mass spectrometry was used for identifying the volatile components. The analysis was carried out with a Shimadzu GC-17A linked with a Shimadzu QP-5050 at an MS ionization voltage of 70 eV, accelerating voltage of 1500 V, and ion source temperature of 250°C. The GC column was DB-Wax fused-silica capillary type (60 m x 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA). The column temperature was programmed from 70°C (2 min hold) to 100°C at a rate of 2°C/min. The column was cleaned by heating to 230°C before each run. The injector temperature was 250°C, and helium was used as the carrier gas at a flow rate of 0.8 ml/min. An oil sample of 0.2 μL was injected at a split ratio of 1:50.

Identification of the Components

Each component was initially identified by the GC retention index and the NIST library connected to the QP-5050 mass spectrometer, as described in previous papers. (10,11) A JNM-LA400 spectrometer (Jeol, Tokyo) was employed for recording the ^{13}C -NMR spectra, CDCl_3 being used as a solvent.

Determination of the Isotope Ratio.

The following 10 monoterpene compounds were examined in the determination of isotope ratios: α -pinene, β -pinene, sabinene, myrcene, α -phellandrene, α -terpinene, limonene, γ -terpinene, β -phellandrene and terpinolene. Selected ion monitoring (SIM) of GC-MS was performed in order to estimate the intensities of the ion peaks of each molecule (m/z 136) and of its isotope (m/z 137). The total intensity of each compound was

regulated to achieve about 8.0×10^7 in MS, the mass spectrometry scanning interval being 0.1 sec. The isotope ratio (Ir) of each peak was calculated by the following equation:

$$\text{Ir} = (\text{Intensity of } m/z \text{ 137 peak}) / (\text{Intensity of } m/z \text{ 136 peak}) \times 100$$

where the intensity is the mean value from quintuplicate measurements.

Synthesis of Esters

The ^{13}C -labeled esters were synthesized by a conventional method. (12) A solution of 9 mmol of butanol, pentanol, hexanol, heptanol, octanol, nonanol or decanol in benzene was mixed with 82 mmol $1\text{-}^{13}\text{C}$ -acetic acid. The reaction mixture was refluxed for 1.5 hr with a small amount of *p*-toluene sulfonic acid. The reaction proceeded almost perfectly, and mixtures containing the esters of the 5-10 carbon number at 2.6-3.8% (w/w) were obtained (Table I). The synthesized ^{13}C -labeled esters were determined by GC and GC-MS. Non-labeled esters were synthesized as well.

Table I. Components of the ^{13}C -Labeled Ester Mixture

<i>Compound</i>	<i>Conc.</i> % (w/w)	<i>Molecular</i> <i>weight</i>
Pentyl acetate	2.36	131.2
Hexyl acetate	2.64	145.0
Heptyl acetate	2.96	159.2
Octyl acetate	3.23	173.3
Nonyl acetate	3.54	187.3
Decyl acetate	3.80	201.3

Synthesis of Limonene

4-Acetyl-1-methylcyclohexene was synthesized by the method described by Lutz *et al.* (13) and Fray *et al.* (14) Ninety mmol of SnCl_4 was added to 120 mL of benzene while stirring at 3°C , and then 550 mmol of isoprene was added. The reaction mixture was added to 500 mmol of methyl vinyl ketone over a 15-min period, and then stirred continuously for 2 hr at $5\text{-}10^\circ\text{C}$. The mixture was successively washed with an NaCl aq. solution and KOH aq. solution, and dried with sodium sulfate. After drying, the mixture was evaporated to 31.4 g (a yield of 45.4%). Identification was carried out by GC and GC-MS. The purity of 4-acetyl-1-methylcyclohexene was 96% by GC, containing 4% of 3-acetyl-1-methylcyclohexene.

¹³C-Labeled limonene, 1-methyl-4-(1-methyl-(2-¹³C)-ethenyl)-cyclohexene, was synthesized by means of the Wittig-reaction. (15). The labeling reagent, 4.9 mmol ¹³CH₃P(C₆H₅)₃I, was added at 20°C over 5 min to a solution of 4.9 mmol NaH in 5mL of DMSO, which had been prepared while stirring at below 70°C, and the mixture stirred for 20 min. After adding 5.0 mmol of acetyl-1-methylcyclohexene at 20°C over 5min, the reaction mixture was stirred for 2.5 hr at room temperature. Extraction was performed with pentane and, after drying with sodium sulfate, the solvent was removed to obtain the final product of 0.14g (a yield of 20.9%).

Results and Discussion

Accuracy of the Isotope Ratio by Ordinary GC-MS

In principle, it is possible to obtain the isotope ratio from MS data. The authors have previously shown, in fact, a practical use for the isotope ratio from mass spectrometry. (10) However, more detailed analytical conditions need to be found to achieve more precise measurement. The intensity of each molecular ion peak is not very strong. In addition, the isotope peak is approximately 10% of that of the molecular ion peak in the case of monoterpene hydrocarbons in citrus essential oils.

The repeatability of the *I_r* values for various monoterpene hydrocarbons was examined. The concentrations of the standard samples for GC-MS of α-pinene, β-pinene, sabinene, myrcene, α-phellandrene, α-terpinene, limonene, γ-terpinene and terpinolene were within the range of 0.5-1.8% (w/w). The *I_r* value for each monoterpene hydrocarbon was plotted in Fig. 1, where the coefficient of variation (CV) is also presented. These *I_r* values only seem to vary over a narrow range, since their CVs were not more than 0.61% (limonene).

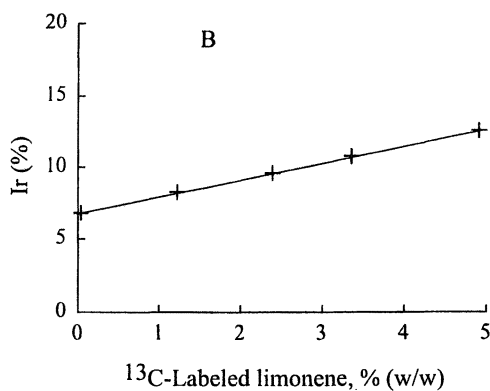
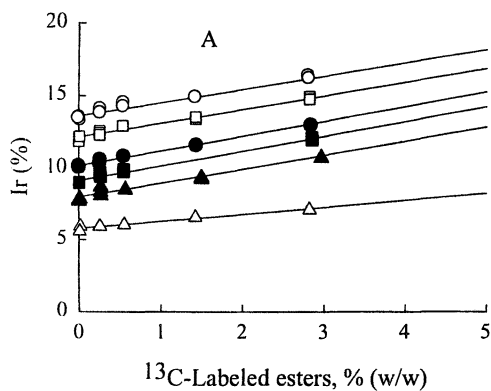
4-Acetyl-1-methylcyclohexene had a purity of 95% by GC, and contained 5% 1-methyl-3-(1-methyl-(2-¹³C)-ethenyl)-cyclohexene. It was confirmed that the δ 108.30 (C-10) signal was increased. The *m/z* and peak intensity (%) data for the fragment in the mass spectrum were as follows: MS (non-labeled limonene) *m/z* 136 (18, M⁺), 107 (19), 94 (27), 93 (67), 92 (23), 78 (30), 68 (100), 67 (78), 52 (29); MS (¹³C-labeled limonene) *m/z* 137 (18, M⁺), 108 (19), 95 (24), 94 (35), 93 (51), 79 (19), 68 (100), 67 (52), 52 (27). The δ values from the ¹³C-NMR spectrum of each carbon in the ¹³C-labeled limonene were as follows: δ 133.75 (C-1), δ 27.92 (C-2), δ 30.59 (C-3), δ 41.08 (C-4), δ 30.81 (C-5), δ 120.67 (C-6), δ 23.45 (C-7), δ 150.25 (C-8), δ 20.80 (C-9), δ 108.30 (C-10).

The relationship between the ratio of the isotope peak intensity to the molecular mass peak intensity (I_r) and the isotopic ratio of ^{13}C were then examined. ^{13}C -labeled esters and limonene were added to non-labeled compounds, and the I_r values were measured. As shown in Fig. 2, a strong linear relationship was observed between the I_r value and the ^{13}C isotopic ratio. The R^2 value for the esters was greater than 0.96, except for decyl acetate. The accuracy with decyl acetate was rather low ($R^2=0.85$), because the intensity of the molecular ion peak followed the normal tendency of decreasing with increasing molecular weight. The difference in the intercept and gradient between octyl acetate and the other acetates may be explained by the difference between the isotope ratio of octanol and those of the other alcohols.

These results show that the reproducibility for determining the peaks of the molecular ion and its isotope by ordinary GC-MS is satisfactory and applicable for practical use. It is expected that this method of isotope analysis can also be applied to organic compounds which have a wide range of molecular weight.

Ir Analysis of Monoterpene Hydrocarbons from Citrus Essential Oils

The isotope ratio analysis was carried out on various kinds of citrus essential oils, including commercial CPO of five lemon oils and five lime oils, an artificial lemon flavor product, a lemon CPO and ten yuzu CPO from Japan. The isotope effect was found to be influenced by exogenous factors such as differences in location, climate and cultivation conditions. (2,6) When the I_r s of monoterpene hydrocarbons were divided by the I_r of one compound, limonene, of each essential oil (modified I_r), the influence on isotope discrimination by CO_2 fixation was eliminated. (6) This calculation will result in I_r patterns based on secondary metabolites, showing the specific pattern of each plant species. As shown in Fig. 3, a characteristic fingerprint was obtained for each citrus species. The composition of citrus essential oils generally depends on the variety or species. Thus, the six monoterpene hydrocarbons were selected as common constituents of the three kinds of CPO for performing the isotope ratio analysis. These fingerprints of lemon, lime and yuzu were clearly distinct, the fingerprint band of lime being broader than that of yuzu and lemon. This may have been due the fact that the lime oil sample was a mixture of products from various localities, varieties and/or distillate oil from juice. The fingerprint of yuzu CPO agreed with the one reported previously. (10)



- ▲ : Pentyl acetate $y = 8.053 + 0.961x$ $R^2 = 0.958$
 ■ : Hexyl acetate $y = 9.196 + 1.016x$ $R^2 = 0.984$
 ● : Heptyl acetate $y = 10.224 + 1.000x$ $R^2 = 0.992$
 △ : Octyl acetate $y = 5.871 + 0.484x$ $R^2 = 0.980$
 □ : Nonyl acetate $y = 12.19 + 0.949x$ $R^2 = 0.976$
 ○ : Decyl acetate $y = 13.63 + 0.917x$ $R^2 = 0.849$
 + : Limonene $y = 10.047 + 0.866x$ $R^2 = 1.000$

Figure 2. *Ir values of the ^{13}C -labeled compounds.*
A: ^{13}C -labeled esters
B: ^{13}C -labeled limonene

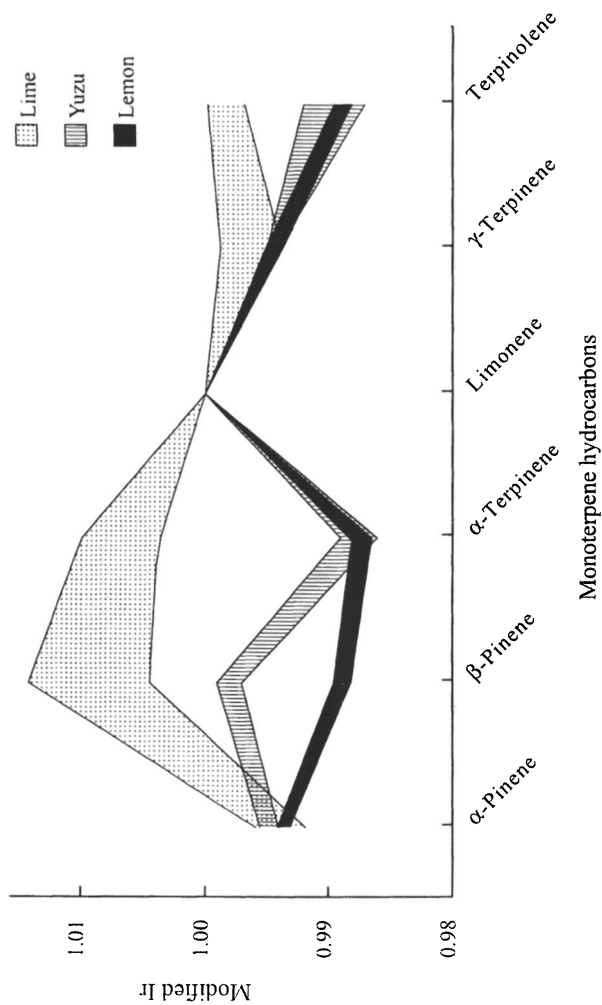


Figure 3. Modified Ir values of the monoterpene hydrocarbons from citrus oils.

Multivariate Analysis.

The modified Ir data for monoterpene hydrocarbons from the three species of citrus oils were subjected to a multivariate analysis. According to principal component (PC) analysis, the citrus essential oils examined were clearly discriminated, with 93.3% of the accumulation contribution ratio of both PC1 and PC2 (Fig. 4A). As shown in Fig. 4B, β -pinene (PC1, 0.530 · PC2, -0.80), α -terpinene (0.685 · 0.062) and β -phellandrene (0.324 · 0.810) were large for the absolute value of the eigenvector of monoterpene system hydrocarbons, followed by terpinolene (0.367 -0.010). These compounds greatly contributed to the modified Ir discrimination of the citrus essential oils.

The cluster analysis is shown Fig. 5. All of the essential oils, except for lime oil E, were classified into the species clusters. As stated above, the most likely reason for lime oil being the outlier was that the sample was a mixture of oils. Regarding the yuzu oils, it is noteworthy that the essential oils from Yamagata, Kochi, Shizuoka and Tokushima prefectures, which each had a high sensory evaluation, were classified into the same cluster, which is in accordance with the findings in our previous report. (10) The artificial lemon flavor essence and the lemon CPO from Wakayama Prefecture in Japan were classified into the same cluster as the commercial lemon samples. This clearly shows that the artificial lemon flavor essence is based on a natural lemon CPO starting material.

Summary

These results suggest that, in addition to an enantiomeric analysis, (16,17) this analytical method can be applied to evaluate the genuineness of essential oils. Furthermore, it is expected that this technique of isotope ratio analysis will be applicable to evaluate the origin of citrus essential oils and their products, and also to specify organic compounds from natural resources.

References

1. Park, R.; Epstein, S. *Geochim. Cosmochim. Acta*, **1960**, *21*, 110-126.
2. O'Leary, M. H. *Phytochemistry*, **1981**, *20*, 553-567.
3. Shaw, P. E. *J. Agric. Food Chem.*, **1979**, *27*, 246-257.
4. Sawamura, M.; *Recent Research Developments in Agricultural and Food Chemistry*, Pandalai, S G. Ed.; Research Signpost, Trivandrum, India, 2000, Vol. 4, pp.131-164.

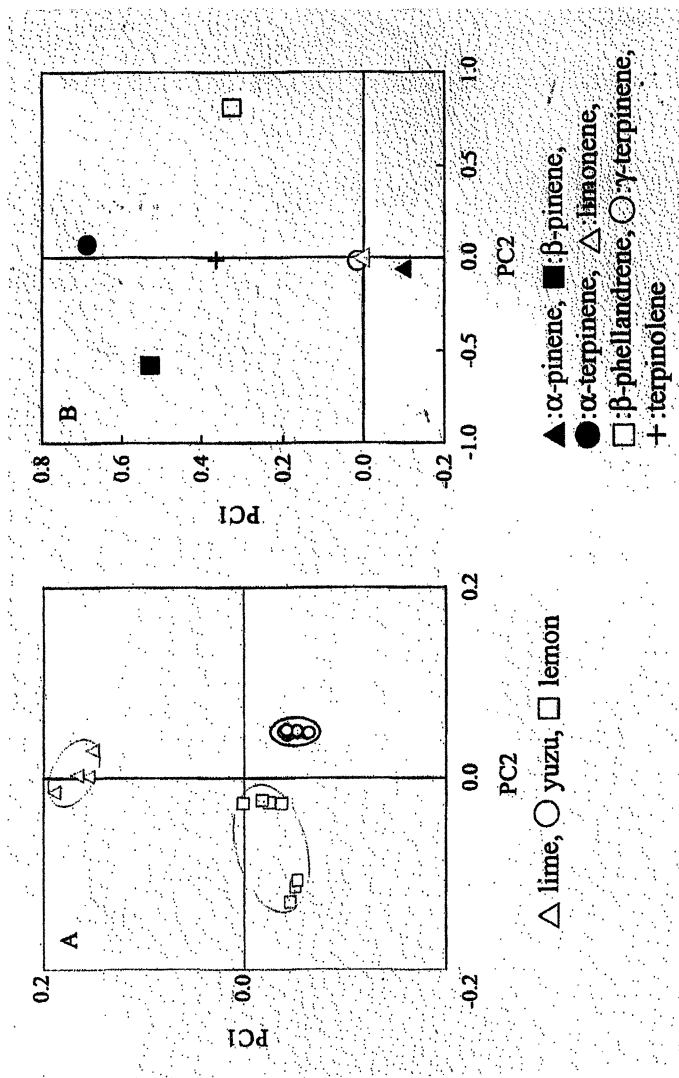


Figure 4. Principal Component Analysis of the modified Ir values of the monoterpane hydrocarbons from citrus oils.

A: Principal component scores of citrus oils

B: Characteristic vectors of monoterpane hydrocarbons

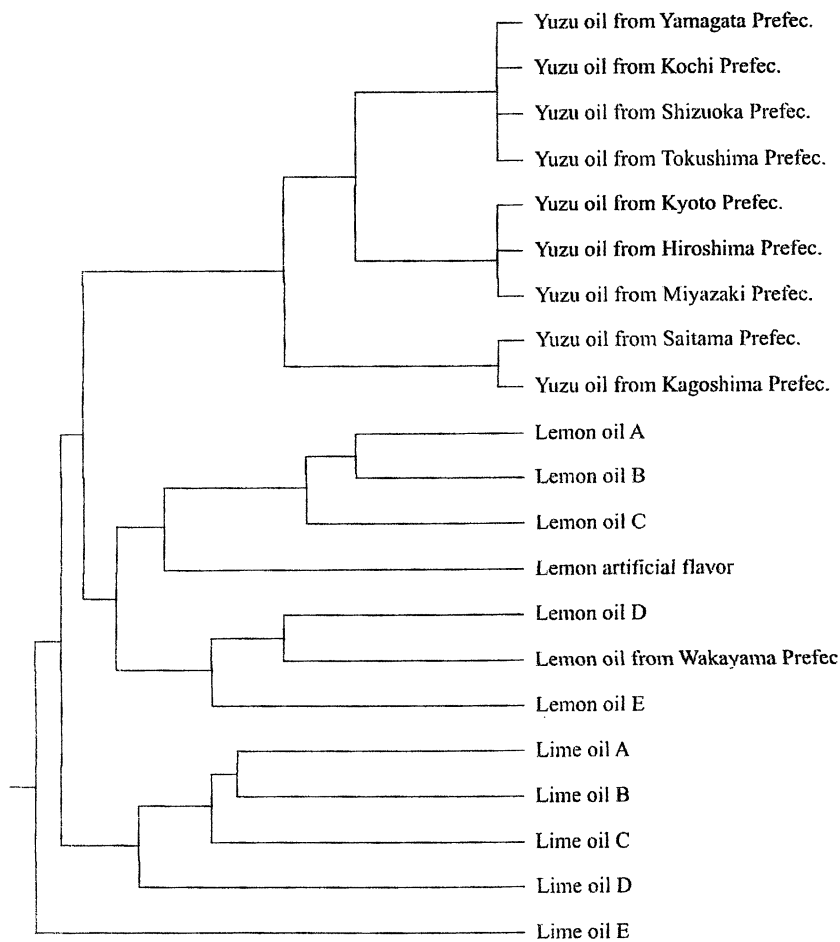


Figure 5. Cluster analysis of the modified I_r values of the monoterpene hydrocarbons from citrus oils.

5. Faulharber, S.; Hener, U.; Mosandl, A. *J. Agric. Food Chem.*, **1997**, *45*, 2579-2583.
6. Faulharber, S.; Hener, U.; Mosandl, A. *J. Agric. Food Chem.*, **1997**, *45*, 4719-4725.
7. Faber, B.; Krause, B.; Dietrich, A.; Mosandl, A. *J. Essent. Oil Res.*, **1995**, *7*, 123-131.
8. Frank, C.; Dietrich, A.; Kremer, U.; Mosandl, A. *J. Agric. Food Chem.*, **1995**, *43*, 1634-1637.
9. Hener, U.; Faulhaber, S.; Kreis, P.; Mosandl, A. *Pharmazie*, **1995**, *50*, 60-62.
10. Sawamura, M.; Ito, T.; Une, A.; Ukeda, H.; Yamazaki, Y. *Biosci. Biotechnol. Biochem.*, **2002**, *12*, 2622-2629.
11. Choi, H. S.; Sawamura, M. *Biosci. Biotechnol. Biochem.*, **2001**, *65*, 48-55.
12. Weissberger, A.; Kibler, C. *J. Org. Synth.*, **1943**, *2*, 610-613.
13. Lutz, E. F.; Bailey, G. M. *J. Amer. Chem. Soc.*, **1964**, *86*, 3899-3901.
14. Fray, G. I.; Robinson, R. *J. Amer. Chem. Soc.*, **1961**, *83*, 249.
15. Reichert, S.; Fischer, D.; Asche, S.; Mosandl, A. *Flavour Fragr. J.*, **2000**, *15*, 303-308.
16. Cotroneo, A. *Flavour Fragr. J.*, **1992**, *7*, 15-17.
17. Mitiku, S. B.; Sawamura, M.; Njoroge, S. M.; Koaze, H. *J. Essent. Oil Res.*, **2002**, *14*, 196-202.

Chapter 8

Characteristic Aroma Components of the Cilantro Mimics

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Aroma profiles of fresh herbs of cilantro (*Coriandrum sativum* L.), culantro (*Eryngium foetidum* L.) and Vietnamese coriander (*Polygonium odoratum* L.) were compared by direct solvent extraction, gas chromatography-olfactometry and aroma extract dilution analysis. Among the three herbs, the key aroma components of *C. sativum* and *E. foetidum* were most similar and consisted of mainly 2-alkenals and n-aldehydes. On the other hand, *P. odoratum* contained mostly decanal and dodecanal, with 2-alkenals being notably absent in this herb. *C. sativum* and *E. foetidum* were found to differ in volatile composition, with (*E*)-2-alkenals from C₉-C₁₆ and decanal being predominant in *C. sativum*, while *E. foetidum* contained mostly (*E*)-2-dodecenal. (*E*)-2-Alkenals, in particular (*E*)-2-dodecenal, were most closely associated with characteristic aromas of *C. sativum* and *E. foetidum* herbs; whereas, decanal and dodecanal were the characterizing components of *P. odoratum* aroma.

Coriander (cilantro, *Coriandrum sativum* L.), culantro (*Eryngium foetidum* L.) and Vietnamese coriander (*Polygonum odoratum* L.) make up a group of fresh culinary herbs collectively known as the cilantro mimics. These herbs possess similar intense fresh-green, soapy and aldehydic notes and are used in very much the same manner in various parts of the world. *C. sativum*, also commonly known as Chinese parsley, is a widely used throughout the world and is especially important in Latin American and Asian cuisine. The fresh leaves of *E. foetidum* closely resemble cilantro in aroma, but the appearance of this herb is very different having long, serrated and thistle-like leaves. *E. foetidum* has long been used in the Far East, Latin America and the Caribbean. *P. odoratum* (rau ram) is often used interchangeably with cilantro herb or mint in Southeast Asia, principally in Vietnamese cuisine.

There have been a number of reports on the essential oil composition of the above mentioned cilantro mimics; however, there is limited information on the characteristic aroma-active components of the fresh herbs. The following investigation was undertaken to identify and compare the characteristic aroma components of fresh leaves of *C. sativum*, *E. foetidum*, and *P. odoratum*. The essential oils from seeds of these plants differ significantly from that obtained from the green, leafy (herb) parts of the plant (the essential oil of the seeds of *C. sativum* is contains mostly linalool) and will not be addressed in this investigation.

Experimental Procedures

Herbs were cultivated from commercial seeds at the University of Illinois (Urbana, IL). Cold direct solvent extraction of the fresh herbs was performed within one hour of harvest using a modification of the procedure previously described (1,2), except that 10 μL of an internal standard solution (containing 10 μg of 6-undecanone in methanol) was added to the sample before extraction. Aroma extracts were subjected to a high-vacuum distillation clean-up step using a modified SAFE head (2,3), dried over anhydrous sodium sulfate (10 g) and concentrated to 10 mL under a gentle stream of nitrogen.

Volatile composition (based on percent peak areas) was determined by gas chromatography-mass spectrometry (GCMS). Aroma extract (1 μL) was injected in the cold splitless mode using a programmable temperature vaporizer (PTV) inlet [splitless time 1.0 min; vent flow = 50 mL/min; initial temperature -50°C (0.1 min) to 240°C (10 min final time) at 12°C/s] into an HP6890 GC/5973 GCMS system (Agilent Technologies, Inc., Palo Alto, CA) equipped with a HP-5ms [30 m x 0.25 mm i.d. x 0.5 μm film; Agilent Technologies, Inc.] capillary column. Helium was carrier gas at 1 mL/min constant flow. Oven temperature

was programmed from 35 to 225°C at a rate of 4°C/min with initial and final hold times of 5 and 30 min, respectively.

Aroma-active constituents were determined by gas chromatography-olfactometry (GCO) and aroma extract dilution analysis (AEDA) as previously described (1,2).

Results and Discussion

Essential Oil Composition

Coriander herb (cilantro)

The characteristic aroma of fresh *Coriander sativum* herb is pungent and intensely fresh-green and soap-like. Carlblom (4) was first to study the essential oil composition of *C. sativum* and reported aldehydes (~95%), such as decanal, 2-decenal and 8-methyl-2-nonenal, as the main constituents. Later studies by other groups confirmed the presence of these compounds in *C. sativum* and most agree in that a series of 2-alkenals and saturated aldehydes are the predominant essential oil components of the fresh herb (1,5-10). However, these reports differ considerably in the relative abundances reported for the individual volatile components. Scratz and Quadry (5) reported that 2-tridecenal and decanal were most abundant in the fresh herb during the early stages of plant development. MacLeod and Islam (6) later reported 7-dodecenal (~21%) as the major constituent, but did not report any 2-alkenals. Based on results of other studies, it seems probable that 2-dodecenal was misidentified in that study as 7-dodecenal. In other studies, Potter and Fagerson (7) reported (E)-2-decenal (~46%) as predominant. Lawrence (8) and Mookherjee (9) found mostly alkanals and 2-alkenals as major constituents during plant ontogenesis. Recently, Potter (10) and Cadwallader et al. (1) reported mainly aldehydes (C₁₀-C₁₆), with the 2-alkenals predominating. Potter (10) noted considerable differences in volatile composition between two commercially obtained plants, as well as during ontogenesis for plants propagated in growth chambers. Similarly, differences between 'fresh' and commercial plants were noted by Cadwallader et al. (1).

In the present study, we found a total of thirteen major volatile constituents, having average percent areas >1% of the total, in fresh *C. sativum* herb (Table I). These findings agree with those of Potter and Fagerson (7) and Cadwallader et al. (1), who reported (E)-2-decenal (no. 3), (E)-2-dodecenal (no. 8), (E)-2-tetradecenal (no. 11) and decanal (no. 2) as the most abundant volatiles in *C. sativum* herb.

Culantro herb

The fresh herb of *Eryngium foetidum* L. has a characteristic aroma that very closely resembles *C. sativum* herb. Leclercq et al., (11) reported a total of nineteen constituents in *E. foetidum* of Vietnamese origin, including (*E*)-2-dodecenal (~45.5%) and (*E*)-2-dodecenoic acid (~15.5%) as major constituents,

Table I. Predominant Components of the Essential Oil Obtained from the Herb *C. sativum* L.

No.	Compound	Percent Peak Area*
1	Nonane	1.4
2	Decanal	22.0
3	(<i>E</i>)-2-Decenal	17.4
4	Undecanal	1.4
5	(<i>E</i>)-2-Undecenal	1.9
6	Dodecanal	3.7
7	(<i>Z</i>)-2-Dodecenal	1.4
8	(<i>E</i>)-2-Dodecenal	14.6
9	(<i>E</i>)-2-Tridecenal	3.2
10	(<i>Z</i>)-2-Tetradecenal	2.5
11	(<i>E</i>)-2-Tetradecenal	18.1
12	(<i>E</i>)-2-Pentadecenal	4.5
13	(<i>E</i>)-2-Hexadecenal	1.4

* Average (n=2) percent peak area from GCMS data.

with minor amounts of aldehydes and unsaturated acids. Similarly, Wong et al. (12) reported (*E*)-2-dodecenal (~59.7%) as the predominant essential oil component of Malaysian *E. foetidum*, with low levels of numerous other aldehydes. No acidic volatile components were reported in this study. Recently, Pino et al. (13) reported only 5.7% (*E*)-2-dodecenal among a total of 48 volatile constituents in the hydro-distilled oil of *E. foetidum* herb.

In the present study seven constituents with percent areas near or exceeding one percent were detected, with (*E*)-2-dodecenal (no. 8) comprising over 55% of the total volatiles. The remaining six compounds consisted of several 2-alkenals (nos. 5, 7 and 11), dodecanal (no. 6) and Dodecanoic (no. 14) and (*E*)-2-dodecenoic (no. 15) acids. Our findings compare well with those of Leclercq et al. (11), to a lesser extent with those of Wong et al. (12), and differ greatly from the findings of Pino et al. (13). The presence of Dodecanoic and (*E*)-2-dodecenoic acids is noteworthy since these compounds are unique to *E.*

foetidum and have not been reported as essential oil constituents of either of the other two cilantro mimics, *C. sativum* and *P. odoratum*.

Vietnamese coriander herb

The aroma of the fresh herb of *Polygonum odoratum* is characterized as intensely green, aldehydic, citrus-like and notably lacks the soapy character of *C.*

Table II. Predominant Components of the Essential Oil Obtained from the Herb *E. foetidum* L.

No.	Compound	Percent Peak Area*
5	(<i>E</i>)-2-Undecenal	0.9
6	Dodecanal	5.8
7	(<i>Z</i>)-2-Dodecenal	7.5
8	(<i>E</i>)-2-Dodecenal	55.4
11	(<i>E</i>)-2-Tetradecenal	6.5
14	Dodecanoic acid	4.9
15	(<i>E</i>)-2-Dodecenoic acid	9.3

* Average (n=2) percent peak area from GC-MS data.

sativum and *E. foetidum*. Aliphatic straight-chain aldehydes have been reported as the main volatile constituents of *P. odoratum*, accounting for approximately 80 percent of the oil by weight with the remaining constituents being mainly composed of sesquiterpenes and aliphatic saturated alcohols and hydrocarbons (14,15). Dung et al. (16) reported essentially the same constituents as the above two studies, but indicated the predominance of sesquiterpenes, comprising nearly 45 percent of the essential oil. Results of our analysis of the fresh herb of *P. odoratum* revealed the presence of nine major volatile constituents (Table III). Our findings agree well with those of Potter et al. (14) and Hunter et al. (15). Dodecanal (no. 6) and decanal (no. 2) were in highest abundance, accounting for over 50% of the volatile composition. Undecane (no. 17) and the sesquiterpenes (*E*)-caryophyllene (no. 19), α -humulene (no. 20) and caryophyllene oxide (no. 21) were found at moderately high levels.

Table III. Predominant Components of the Essential Oil Obtained from the Herb *P. odoratum* L.

No.	Compound	Percent Peak Area*
16	Hexanal	0.4
17	Undecane	10.7
18	Nonanal	0.2
2	Decanal	23.2
4	Undecanal	3.0
6	Dodecanal	27.5
19	(<i>E</i>)-Caryophyllene	7.9
20	α -Humulene	5.6
21	Caryophyllene oxide	3.2

* Average (n=2) percent peak area from GC-MS data.

Aroma-Active Components

The above discussion focused mainly on defining the major volatile constituents of the cilantro herbs. Due to their high abundance and overall low threshold values, the n-aldehydes and 2-alkenals are hypothesized to play predominant roles in the characteristic aromas of these herbs, especially nos. **2**, **3**, **8** and **11** in *C. sativum*, and (*E*)-2-dodecenal (no. **8**) in *E. foetidum*. Meanwhile, the volatile composition of *P. odoratum* differs markedly from *C. sativum* and *E. foetidum* in that *P. odoratum* does not contain (*E*)-2-alkenals. Instead, this herb contains mostly n-aldehydes, in particular decanal (no. **2**) and dodecanal (no. **6**), which are most likely the principal contributors to this herb's characteristic aroma. However, it is possible that additional minor constituents are important in the overall aromas of these herbs. In addition, it is not at all apparent by the above results which compounds actually contribute the most to the characteristic aroma of each herb. Therefore, aroma extract dilution analysis (AEDA) was applied to the volatile extracts prepared from *C. sativum*, *E. foetidum*, and *P. odoratum*.

In an earlier study we conducted AEDA for the determination of the predominant aroma-active components of *C. sativum* (*I*), in which we determined that a nonpolar DB-5 column gave superior results during AEDA to a polar DB-WAX column. Therefore, in the present study a DB-5 column was used for AEDA and for the determination of flavor dilution (FD)-factors. For

compound identification purposes (i.e., for calculation of retention indices) we also conducted GCO on concentrated extracts using a DB-WAX column.

Coriander herb (cilantro)

In the present study a total of 19 aroma-active compounds with FD-factors ≥ 9 were detected by AEDA in *C. sativum* solvent extracts (Table IV). The 2-alkenals comprised the majority of the compounds with a total of 10 being detected. (*E*)-2-Decenal (no. 3) and (*E*)-2-dodecenal (no. 8) had the highest FD-

Table IV. Aroma-Active Components Obtained from the Herb *C. sativum* L.

No.	Compound	RI		Odor Description	FD-Factor*
		DB-5	FFAP		
22	(Z)-3-Hexenal	803	1147	Green, cut-leaf	27
23	(Z)-1,5-Octadien-3-one	987	1381	Metallic	9
24	(Z,Z)-3,6-Nonadienal	1100	--	Fresh watermelon	9
25	Linalool	1105	1543	Floral, honeysuckle	27
26	(Z)-2-Nonenal	1149	1505	Stale, hay	27
27	(E)-2-Nonenal	1161	1528	Stale, hay	81
2	Decanal	1209	1507	Green, pungent	729
28	(Z)-2-Decenal	1247	1615	Stale, melon, hay	729
3	(E)-2-Decenal	1267	1648	Soapy, melon	2187
4	Undecanal	1310	1606	Green, pungent	81
29	Unknown	1329	--	Musty, fresh green	9
5	(E)-2-Undecenal	1366	1755	Cilantro, soapy	243
30	Unknown	1398	1772	Musty, chlorine	243
6	Dodecanal	1409	1713	Green, pungent	81
7	(Z)-2-Dodecenal	1454	--	Fishy, soapy, green	27
8	(E)-2-Dodecenal	1475	1875	Cilantro, soapy	2187
31	(Z)-2-Tridecenal	1555	--	Fishy	27
9	(E)-2-Tridecenal	1571	1973	Soapy, waxy	729
11	(E)-2-Tetradecenal	1677	2083	Soapy, waxy	243

* Flavor dilution factor determined on DB-5 column.

factors (= 2187) followed by decanal (no. 2), (*Z*)-2-decenal (no. 28) and (*E*)-2-tridecenal (no. 31) with FD-factors of 729. Compounds of moderate potency (FD-factors of 243) included (*E*)-2-undecenal (no. 5), (*E*)-2-tetradecenal (11) and an unknown compound (no. 30) described as musty, chlorine-like. Eleven additional compounds had FD factors between 9 and 81. These included two n-aldehydes (nos. 4 and 6), four 2-alkenals (nos. 7, 26, 27 and 31), three lipoxygenase-derived compounds (nos. 22, 23 and 24), linalool (no. 25), and an unknown compound with a musty, fresh green note (no. 29). Presence of linalool is not surprising since it the major component of *C. sativum* seed oil (9, 17) and has occasionally been reported as a minor compound in the fresh herb (9, 18).

Based on these results (*E*)-2-alkenals, especially nos. 3, 5, 8 and 9, possessing characteristic green, soapy, and cilantro-like odor qualities are the predominant character-impact aroma components of *C. sativum*. A series of (*Z*)-2-alkenals also were tentatively identified. To our knowledge these (*Z*) isomers have not been previously reported as essential oil constituents of the herb *C. sativum*. The odor characteristics of the (*Z*)-2-alkenals differed from the (*E*) isomers in that the (*Z*) isomers [e.g. (*Z*)-2-decenal, no. 28; (*Z*)-2-dodecenal, no. 7; and (*Z*)-2-tridecenal, no. 31] were described as stale or fishy, whereas the corresponding (*E*) isomers were considered to have green, soapy and cilantro-like notes. The two unknown compounds (nos. 29 and 30) with fresh green, musty and chlorine-like aroma notes also may contribute to the overall aroma of *C. sativum* herb.

Culantro herb

In *E. foetidum* a total of 13 aroma-active constituents with FD-factors ≥ 9 were detected. These consisted of two n-aldehydes (nos. 2 and 6), seven 2-alkenals (nos. 3, 5, 7, 8, 9, 11, 28), (*Z*)-3-hexenal (no. 22) and three unknown compounds (nos. 29, 30 and 32)(Table V). All of these compounds were detected in *C. sativum* with the exception of an unknown compound (no. 32) having a spicy, herbaceous note. (*E*)-2-Dodecenal was by far the predominant aroma component with an FD-factor of 2187. Unknown compound no. 30 had the second highest FD factor (=243). The musty, chlorine-like character of this compound may be important in the overall aroma of *E. foetidum* herb. Other aroma contributors include compounds with FD factors from 27 to 81, such as nos. 5, 6, 7, 11, 22, and 29. Despite having an FD factor of 27, (*Z*)-3-hexenal (no. 22) was probably derived via lipoxygenase action during sample preparation and may not be a characteristic aroma component of essential oil of *E. foetidum* herb.

Table V. Aroma-Active Components Obtained from the Herb *E. foetidum* L.

No.	Compound	RI		Odor Description	FD-Factor*
		DB-5	FFAP		
22	(Z)-3-Hexenal	803	1147	Green, cut-leaf	27
2	Decanal	1209	1507	Green, pungent	9
28	(Z)-2-Decenal	1247	1615	Stale, melon, hay	9
3	(E)-2-Decenal	1267	1648	Soapy, melon	9
29	Unknown	1329	--	Musty, fresh green	27
5	(E)-2-Undecenal	1366	1755	Cilantro, soapy	81
30	Unknown	1398	1772	Musty, chlorine	243
6	Dodecanal	1409	1713	Green, pungent	81
7	(Z)-2-Dodecenal	1454	--	Fishy, soapy, green	27
8	(E)-2-Dodecenal	1475	1875	Cilantro, soapy	2187
9	(E)-2-Tridecenal	1571	1973	Soapy, waxy	3
11	(E)-2-Tetradecenal	1677	2083	Soapy, waxy	27
32	Unknown	1714	2276	Spicy, herbaceous	9

* Flavor dilution factor determined on DB-5 column.

Vietnamese coriander herb

A total of eight aroma-active compounds were detected in solvent extracts of *P. odoratum* (Table VI). Decanal (no. 2) and dodecanal (no. 6) were detected at high FD factors followed by undecenal (no. 4), (Z)-1,5-octadien-3-one (no. 23), hexanal (no. 16), and an unknown compound (no. 33) with a spicy, green note (Table VI). Compounds found at moderate odor intensities included (E,Z)-2,6-nonadienal (no. 34) and two unknown compounds (no. 33 and 35). These findings indicate the overall predominance of decanal and dodecanal as expected from the GCMS results. Although compounds nos. 16, 23 and 34 had relatively high FD factors, their presence is indicative of lipoxygenase activity.

Comparison of cilantro mimics

Results of the present study confirm that n-aldehydes and 2-alkenals are the major volatile constituents of the cilantro mimics and that these herbs differ primarily in the relative levels of these compounds. The essential oils of *C.*

**Table VI. Aroma-Active Components Obtained from the Herb
P. odoratum L.**

No.	Compound	RI		Odor Description	FD-Factor*
		DB-5	FFAP		
16	Hexenal	803	1147	Green, cut-leaf	27
23	(Z)-1,5-Octadien-3-one	987	1381	Metallic	81
33	Unknown	1043	--	Green, spicy	27
34	(E,Z)-2,6-Nonadienal	1153	1760	Cucumber	27
2	Decanal	1209	1507	Green, pungent	243
4	Undecanal	1310	1606	Green, pungent	81
6	Dodecanal	1409	1713	Green, pungent	243
35	Unknown	1432	--	Musty, green	9

* Flavor dilution factor determined on DB-5 column.

sativum and *E. foetidum* are mainly dominated by 2-alkenals, with lesser amounts of n-aldehydes present. These two herbs differ from one another primarily in that (*E*)-2-dodecenal is by far the major volatile constituent of *E. foetidum*, whereas in *C. sativum* a series of (*E*)-2-alkenals from C₉-C₁₆ predominate. The volatile composition of *P. odoratum* differs markedly from *C. sativum* and *E. foetidum*. Most notably *P. odoratum* does not contain (*E*)-2-alkenals and the n-aldehydes decanal and dodecanal, as well sesquiterpenes, are predominant volatile constituents. For the most part, our findings agree well with previous reports on the essential oil compositions of these herbs. Any discrepancies between our findings and those of previous studies may be due to inherent differences between plant source materials, and are likely due to differences in procedures used to isolate the volatile constituents. In the present study, we applied direct solvent extraction under mild conditions in order to obtain more representative aroma extracts, whereas in the majority of previous studies hydro-distillation was used.

Based on the results of AEDA some general conclusions may be drawn about the characteristic aroma components of each herb. In most cases, the predominant aroma-active components determined for each herb follow closely its volatile aldehyde profile. With respect to its essential oil profile and the number of aroma-active constituents, *P. odoratum* herb may be the simplest of the herbs studied. In this herb the n-aldehydes decanal and dodecanal were the predominant aroma-active components, while the sesquiterpene components

were not detected by AEDA despite their relatively high abundance. Aside from dodecanoic (no. 14) and (*E*)-2-dodecenoic (no. 15) acids, all of the major volatile constituents of *E. foetidum* were detected with high FD-factors during AEDA. In this herb, (*E*)-2-dodecenal (no. 8) could be considered the key or characterizing aroma component. *C. sativum* was similar to *E. foetidum* in that predominant aroma components consisted of its major volatile constituents. *C. sativum* has the most complex aroma profile among the cilantro mimics. Our results demonstrate that this herb contains numerous aroma-impact compounds, namely n-aldehydes (nos. 2, 4 and 6), (*E*)-2-alkenals (nos. 3, 5, 8, 9, 11 and 27), (*Z*)-2-alkenals (nos. 7, 28 and 31) and an unknown compound (no. 30). The (*E*)-2-alkenals (nos. 3, 5, 8, 9, and 11) which possess characteristic green, soapy, and cilantro-like aromas are particularly important in the overall aroma of *C. sativum* herb. Among these, (*E*)-2-dodecenal (no. 8) is believed to be the predominant cilantro-like aroma component in *C. sativum*.

References

1. Cadwallader, K.R.; Surakarnkul, R.; Yang, S.-P.; Webb, T.E. In *Flavor Chemistry of Ethnic Foods*; Shahid, F.; Ho, C.-T., Eds.; Kluwer Academic/Plenum Publishers: New York, 1999, Chpt. 7, pp. 77-84.
2. Pojjanapimol, S.; Chaiseri, S.; Cadwallader, K.R. In *Handbook of Flavor Characterization: Sensory Analysis, Chemistry and Physiology*; Diebler, K.D.; Delwiche, J., Eds.; Marcel Dekker, Inc.: New York, 2004, Chpt. 15, pp. 217-230.
3. Engel, W.; Bahr, W.; Schieberle, P. *Eur. Food Res. Technol.* **1999**, *209*, 237-241.
4. Charlblom, A.J. *J. Prakt. Chem.* **1936**, *144*, 225-241 (*Chem. Abstr.* **1936**, *30*, 3772).
5. Scratz, E.; Qadry, S.M.J.S. *Planta Med.* **1966**, *14*, 436-442.
6. MacLeod, A.J.; Islam, R. *J. Sci. Food Agric.* **1976**, *27*, 721-725.
7. Potter, T.L.; Fagerson, I.S. *J. Agric. Food Chem.* **1990**, *38*, 2054-2056.
8. Lawrence, B.M. In *Biogenesis of Aromas*; Parliment, T.H.; Croteau, R., Eds.; ACS Symposium Series 317; American Chemical Society: Washington, DC, 1986, Chpt. 27, pp. 363-369.
9. Mookherjee, B.D.; Wilson, R.A.; Trenkle, R.W.; Zampino, M.J.; Sands, K. In *Flavor Chemistry: Trends and Developments*; ACS Symposium Series 338; American Chemical Society: Washington, DC, 1989, Chpt. 14, 176-187.
10. Potter, T.L. *J. Agric. Food Chem.* **1996**, *44*, 1824-1826.

11. Leclercq, P.A.; Dung, N.X.; Lo, V.N.; Toanh, N.V. *J. Essent. Oil Res.* **1992**, *4*, 423-424.
12. Wong, K.C.; Feng, M.C.; Sam, T.W.; Tan, G.L. . *J. Essent. Oil Res.* **1994**, *6*, 369-374.
13. Pino, J.A.; Rosado, A.; Fuentes, V. *J. Essent. Oil Res.* **1997**, *9*, 467-468.
14. Potter, T.L.; Fagerson, I.S.; Craker, L.E. *Acta Hortic.* **1993**, *344*, 305-311.
15. Hunter, M.V.; Brophy, J.J.; Ralph, B.J.; Bienvenu, F.E. *J. Essent. Oil Res.* **1997**, *9*, 603-604.
16. Dung, N.X.; Hac, L.V.; Leclercq, P.A. *J. Essent. Oil Res.* **1995**, *7*, 339-340.
17. Gil, A.; de la Fuente, E.B.; Lenardis, A.E.; Lopez Pereira, M.; Suarez, S.A.; Bandoni, A.; van Baren, C.; Di Leo Lira, P.; Ghersa, C.M. *J. Agric. Food Chem.* **2002**, *50*, 2870-2877.
18. Fan, X.; Sokorai, K.J.B. *J. Agric. Food Chem.* **2002**, *50*, 7622-7626.

Chapter 9

GC–O and GC–FID Comparison between Early-Mid Season and Valencia Orange Essence Oil

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Sources of perceived quality differences between Valencia orange essence oil and Early-Mid orange essence oil were examined using GC-FID/Olfactometry. The major compositional components in both orange essence oils were terpenes ((+)-limonene) and sesquiterpenes (valencene). The most intense aroma compounds in Valencia essence oil included: wine lactone (dimethyl tetrahydrobenzofuranone), linalool, ethyl butyrate, t-4,5-epoxy-(E)-2-decenal, 1,8-cineole and ethyl heptanoate. The Early-Mid oil intense odorants were: linalool, β -damascenone, E-2-octenal, trans-4,5-epoxy-(E)-2-decenal, octanal, acetaldehyde, an unidentified limonene impurity and myrcene. Sensorally, Valencia oil was described as sweet, orange, with slight warm spice, fatty and green undertone aromas. The Early-Mid oils exhibited green, fatty, slight fruity aromas with only a hint of orange character. Critical differences were associated with five aldehydes and two ketones.

Introduction

Much of the flavor of oranges comes from either the peel oil or from the volatiles recovered from the commercial concentration of orange juice. In the latter process the volatiles collected in the initial condensation stages are collected and allowed to separate into oil and water phases. The oil phase is called orange essence oil and the water phase is known as water phase essence. Essence oil is a highly prized flavoring product. It typically requires over 40,000 Kg of fresh Valencia oranges to produce 3.2 Kg of essence oil (1). Orange essence oil is a complex mixture whose volatile composition has been extensively studied since the advent of gas chromatography (2-5). Early analyses identified and quantified less than 30 of the most abundant components whose recombination did not match the sensory characteristics of the original oil (6). Later studies (7) identified 41 volatiles in essence oil from overripe oranges. Recent studies coupling high resolution GC with ion trap mass spectrometry have increased the number of identified compounds to 95 (8). Since replicating the major components in orange oil did not duplicate its sensory characteristics, GC-olfactometric studies were carried out to determine which components were actually responsible for the characteristic aroma of orange oil. These sensory directed studies (8) identified 55 aroma active compounds in orange essence oil using a combination of mass spectral and retention index data. The most intense aromas were produced by octanal, wine lactone, linalool, decanal, β -ionone, citronellal, and β -sinensal. Potent aroma components reported for the first time in orange essence oil included: E-2-octenal, 1-octen-3-ol, Z-4-decenal, E,E-2,4-nonadienal, guaiacol, γ -octalactone, and m-cresol. Less than 4% of the oil's total mass was responsible for all the observed aroma activity.

In Florida there are essentially two seasons for the orange crop. The early season usually begins in November and ends in January. These early ripening cultivars include primarily Hamlin and Parson Brown. The Valencia season begins in late March and continues through June. Commercial orange essence oil consists primarily of Valencia oil as the early season oil is often not collected as it is considered to be of lower quality. Valencia oranges take the longest time to mature compared to other cultivars and produce the highest quality juice. The purpose of this study was to examine the aroma active components in early season orange essence oil and compare it with those from Valencia oranges in order to determine what aroma components might be responsible for the perceived quality difference.

Materials and Methods

Materials

Samples of Early-Mid and Valencia essence oils were obtained from a citrus processing plant in Lake Wales, Florida on three different processing days. The samples were diluted with ethyl ether (1:80) and 20 μL of 2000 ppm of 4-heptadecanone was added as internal standard to 1.0 mL of the diluted oil. A 0.1 μL aliquot was injected into the GC.

Gas Chromatography

Volatile constituents were separated using a HP-5890A GC (Palo Alto, CA) with a flame ionization detector (FID) and either a Zebron ZB-5 column (30 m x 0.32 mm i.d. x 0.50 mm) from Phenomenex (Torrance, CA) or a DB-Wax column (30 m x 0.32 mm i.d. x 0.50 mm) from J & W Scientific (Folsom, CA) was used. Oven temperature for the ZB-5 column was programmed from 40 to 265°C at 7°C/min with a holding time of 5 min. Samples were injected in the splitless mode. Initial oven temperature for the DB-Wax column was 40°C, and then increased to 240°C at 7°C/min with a holding time of 5 min. Injector temperature was maintained at 220°C and detector temperature at 250°C.

GC-Olfactometry

The GC effluent was split between the FID and an olfactometer (DATU, Geneva, NY) with a humidified air volume of 1.2 L/min. A time-intensity approach was used to evaluate odor quality and intensity at the sniff port under GC conditions described previously. Assessors rated aroma intensity (0-15 scale) continuously throughout the chromatographic separation process using a linear potentiometer. Retention times and verbal descriptors were recorded to permit aroma descriptors to be coupled with computerized aroma time-intensity plots. Two trained assessors evaluated the sample in duplicate, thus producing four individual time-intensity aromagrams. Average intensity from the four runs was calculated for each odorant, if no peak was detected a value of zero was assigned. An averaged time-intensity aromagram was constructed by plotting average intensity versus retention time.

Chromatograms and aromagrams were recorded and integrated using ChromPerfect version 5.0.0, Justice Laboratory Software (Justice Innovations, Inc., Palo Alto, CA, USA). Peaks were identified using Kovats retention indices, calculated using retention time data from a series of alkane standards (C5-C25) run under the same chromatographic conditions.

Sensory Description

Three sensory judges with over 45 years combined citrus experience described the aroma characteristics of the two types of orange essence oil. The undiluted oil was presented on perfumers wands and free choice descriptors were allowed.

Results and Discussion

Sensory Characteristics

The consensus aroma description of the Valencia essence oil was that of a prominent, sweet, pleasant orange aroma. It also possessed warm, spicy, fatty and green undertones. In comparison, the aroma of the Early-Mid essence oil was described as green, fruity and fatty with a weak orange character. Both oils were produced at the same commercial facility, using identical equipment and procedures during the same season. Therefore, any observed differences should be solely due to the composition of the respective cultivars. In Florida, early and mid season oranges are primarily from Hamlin, Pineapple and Parson Brown cultivars.

Instrumental versus Olfactory Detection

Shown in Figure 1 are two representative chromatograms from Valencia and Early-Mid season orange essence oil. In comparing these two chromatograms it becomes readily apparent that there are no obvious differences between the major volatiles in these two oils. However, even though the major volatiles are very similar, the sensory differences are appreciable. As seen from Figure 1, the major component in both orange essence oils is limonene, which typically comprises 90% of essence oils (δ). In comparing the

aroma activity that would be associated with this peak, it appears that most if not all the aroma activity associated with this huge peak are co-eluted impurities. It has been previously reported that essentially 100% of the aroma activity of citrus products is produced from only 4-5% of the volatiles (9). It appears that the minor volatiles produce most of the aroma activity for orange essence oil as well.

Total Aldehydes versus Specific Aldehydes

Aldehydes are highly reactive and typically have low aroma thresholds. Twelve of the 29 aroma active components observed in this study (Table I) were aldehydes. Approximately 40% of the total aroma impact (obtained from summing all GC-O aroma peak intensities for each oil) of both oils was due to aldehydes. This is perhaps why one of the oldest commercial measurements for oil quality has been total aldehydes (10). Total aroma intensities (total aroma

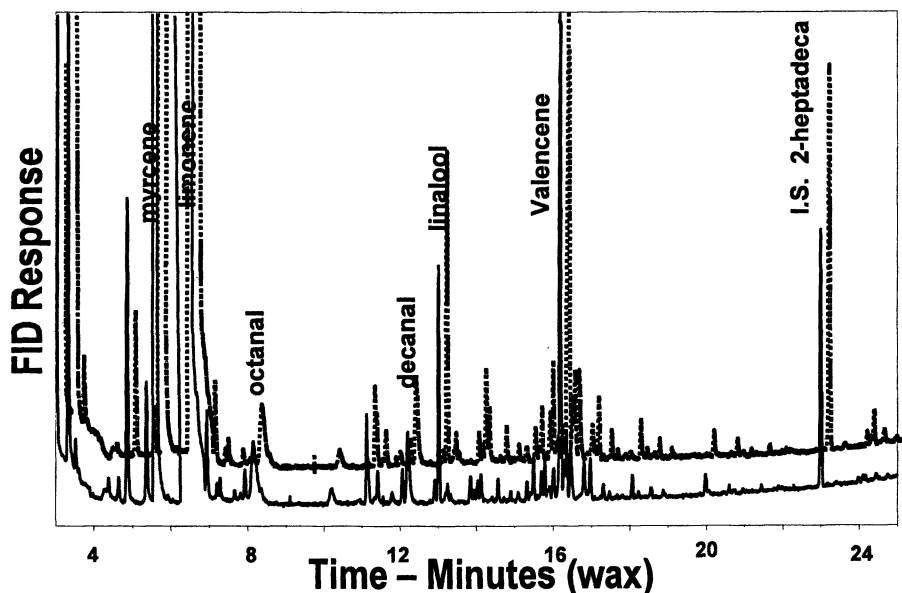


Figure 1. Comparison of gas chromatograms (polar separation) of commercial Valencia orange essence oil (lower solid line) and that of Early-Mid orange essence oil (upper dashed line). The Early-Mid essence oil is offset and shifted slightly for clarity.

peak heights) were 9.2×10^6 for Valencia and 8.9×10^6 for Early-Mid. However, the Valencia oil actually contained a slightly lower percentage (38 versus 39%) of aroma active aldehydes than the Early-Mid oil. As Valencia oil is generally considered to be of higher quality than Early-Mid oil, this data suggests that the specific aldehyde composition may be more important than the total amount of aldehydes.

In comparing the 12 aldehydes and their relative intensities shown in Table I, it is not surprising that seven of the 12 are found in both oils at essentially the same levels. However, the relative intensities of the other five are interesting in that three of the five differing aldehydes are totally absent in the other oil. For example, E-2-octenal (peak 14) was observed only in the Early-Mid season oil whereas Z-2-nonenal (peak 17) and β -sinensal (peak 28) were only found in the Valencia oil. Only two aldehydes, namely acetaldehyde (peak 1, with possible co-elution of ethyl propanoate) and nonanal (peak 13) had appreciable intensity differences. It is quite possible that the major aroma quality differences between these two oils is due to the marked differences in these five aldehydes. β -sinensal has long been considered a major quality factor in orange oils (11) and the lack of this positive aroma component in the Early-Mid oil may explain in part the lower aroma quality of this oil.

Most Intense Odorants

The 10 most intense aroma compounds in the Valencia oil in order of decreasing intensity were wine lactone, linalool, ethyl butyrate, t-4,5-epoxy-(E)-2-decenal, 1,8-cineole, ethyl heptanoate, an unknown (LRI = 975), sotolon, myrcene and octanal. Most of these compounds have been reported as positive quality factors in orange juices and oils. Interestingly, the Early-Mid oil has only 5 of its 10 most intense odorants in common with the Valencia oil. The common odorants include linalool, octanal, t-4,5-epoxy-(E)-2-decenal, myrcene and ethyl butyrate. Except for t-4,5-epoxy-(E)-2-decenal, all of these odorants have been considered to be critical orange flavor components (12,13). The remaining intense odorants in the Early-Mid oil were not as intense in the Valencia oil, suggesting that relative proportion or flavor balance is an important consideration. These remaining odorants include: β -damascenone, E-2-octenal, acetaldehyde, dodecanal and a minty, sweet impurity co-eluting with limonene. With the exception of acetaldehyde, these odorants are not usually associated with positive orange flavor.

Table I. Comparison of aroma active components in Valencia and Early-Mid season orange essence oils

<i>No.</i>	<i>Descriptor</i>	<i>LRI (WAX)</i>	<i>Compound</i>	<i>Intensity Valencia</i>	<i>Intensity EarlyMid</i>
1	fresh, alcohol, sweet	945	acetaldehyde	8.2	12.4
2	acid, sulfur	975	unknown	12.1	9.3
3	green	1022	α -pinene	11.3	0.0
4	sweet, fruity	1034	ethyl butyrate	13.2	11.0
5	green, floral, geranium	1147	(Z)-3-hexenal	11.2	10.2
6	earthy, floral	1166	myrcene	11.9	11.9
7	minty, sweet	1207	limonene impurity	11.1	12.0
8	camphoraceous	1222	1,8 cineole	13.1	10.1
9	sweet, fruity, fermented	1240	ethyl hexanoate	8.7	10.9
10	metallic, cilantro, floral	1298	octanal	11.9	12.7
11	metallic	1308	1-octen-3-one	0.0	9.6
12	sweet	1347	ethyl heptanoate	13.1	0.0
13	metallic	1405	nonanal	9.1	5.2
14	soapy, floral, coconut	1439	E-2-octenal	0.0	13.2
15	metallic	1463	1-octen-3-ol	0.0	9.4
16	metallic, floral	1509	decanal	11.9	10.9
17	spicy	1515	Z-2-nonenal	10.6	0.0
18	fabric, floral	1544	E-2-nonenal	11.5	11.0
19	floral, sweet	1553	linalool	13.3	15.0
20	oily, fatty	1703	dodecanal	9.9	11.2
21	lemon, minty	1749	laevo-carvone	9.7	9.4
22	oily, fatty	1821	E,E-2,4-decadienal	10.8	9.9
23	tobacco, old soda	1834	b-damascenone	5.9	13.7
24	lemon grass	1857	geraniol	11.0	7.1
25	metallic	2013	t-4,5-epoxy-(E)-2-decenal	13.2	12.7
26	acid, animal cage	2085	m-cresol	10.5	3.5
27	burnt	2204	sotolon	12.1	10.4
28	woody, sweet	2256	β -sinensal	10.0	0.0
29	oriental perfume	2294	wine lactone	14.9	0.0

Critical Differences

Figure 2 clearly illustrates the major aroma differences between the two oil types. The differences in aroma intensity were calculated by subtracting the aroma intensity for each aroma component in the Early-Mid oil from the corresponding component in the Valencia oil. Those aroma components with the largest difference are presented first. Only those aroma components whose intensities are appreciably different between the two samples are included in

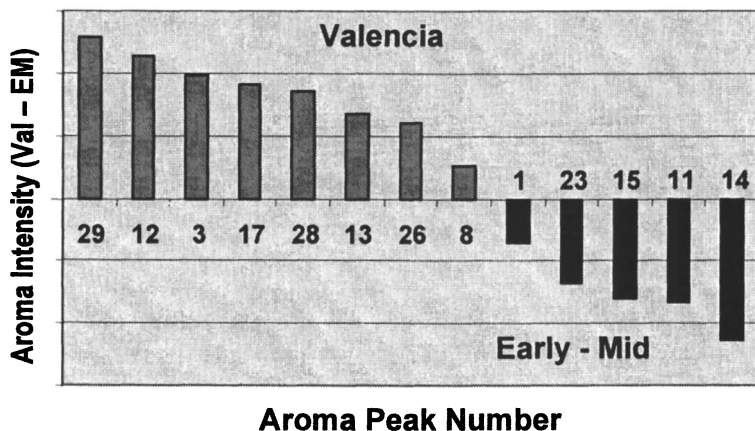


Figure 2. Comparison of the major aroma intensity differences between Valencia and Early-Mid season orange essence oils. Peak identification numbers from Table I.

Figure 2. Those present at appreciably higher intensities in the Valencia will have positive values and those from the Early-Mid oil will have negative values. Noticeably absent from Figure 2 are major orange oil aroma components such as ethyl butyrate, octanal and linalool as they have almost the same intensity in both sample types. As might be expected the five aldehydes discussed earlier (peaks 1, 13, 14, 17 and 28) are present. Other major aroma differences were due to two ketones, wine lactone (peak 29) and β -damascenone (peak 23). Wine lactone was completely absent in the Early-Mid oil as compared to being a major aroma peak in Valencia oil. β -damascenone (peak 23) was one of the most intense aroma peaks in the Early-Mid oil and completely lacking in the Valencia sample.

Conclusion

Some of the data is difficult to explain. For example, m-cresol (peak 26) has a pronounced negative aroma character and was found only in the Valencia oil. Despite these difficulties, much of the observed quality differences between Valencia and Early-Mid season orange essence oils can be explained from the lack of positive aroma components in the Early-Mid season oil and the presence in Early-Mid of aroma components which are not particularly positive.

References

1. Johnson, J. D.; Vora, J. D. *Food Technol* **1983**, *37*, 92-93.
2. Giacomo, A. D.; Rispoli, G.; Tracuzzi, M. L. *Riv. Ital. Essenze-Profumi Piante Offic.-Aromi-Sapono-Cosmet.* **1963**, *45*, 269-281.
3. Attaway, J. A.; Wolford, R. W. *Gas Chromatog., Proc. Symp.* **1965**, *5*, 170-179.
4. Wolford, R. W.; Attaway, J. A. *J. Agric. Food Chem.* **1967**, *15*, 369-377.
5. Coleman, R. L.; Lund, E. D.; Moshonas, M. G. *J Food Sci* **1969**, *34*, 610-611.
6. Shaw, P. E.; Coleman, R. L. *J Agric Food Chem* **1971**, *19*, 1276-1278.
7. Moshonas, M. G.; Shaw, P. E. *J Agric Food Chem* **1979**, *27*, 1337-1339.
8. Hognadottir, A.; Rouseff, R. L. *J. Chromatog. A* **2003**, *998*, 201-211.
9. Lin, J. M.; Rouseff, R. L. *Flavour and Fragrance Journal* **2001**, *16*, 457-463.
10. Braddock, R. J.; Kesterson, J. W. *Citrus Industry* **1977**, *58*, 17-20.
11. Sugisawa, H.; Yamamoto, M.; Tamura, H.; Takagi, N. *J Japan Soc Food Sci Technol* **1989**, *36*, 543-550.
12. Ahmed, E. M.; Dennison, R. A.; Shaw, P. E. *J. Agric. Food Chem.* **1978**, *26*, 368-372.
13. Buettner, A.; Schieberle, P. *J Agric Food Chem.* **2001**, *49*, 2387-2394.

Chapter 10

Sensorial Impact of Sotolon as the “Perceived Age” of Aged Port Wine

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During the barrel aging of Port wine, a number of specific compounds accumulate progressively. One such compound is Sotolon, which imparts a typical aroma, which increases in intensity with age. To correlate Sotolon levels with sensorially determined “age perception”, a simple ranking test was applied to 3 sets of wines: a 4 year old, a 10 year old and a 40:60 blend of these. Increasing quantities of Sotolon were added and wines supplemented with this substance were consistently ranked as older. The Sotolon formation was studied as a function of temperature, pH, SO₂ and dissolved O₂. The synergistic effect of temperature and exposure to high concentrations of O₂ had the highest impact on Sotolon concentration. A 1st order kinetic model was applied to describe both O₂ saturated and non-O₂ supplemented environments on Sotolon formation. The Arrhenius equation accurately described the temperature dependence of the reaction rate constants. The presence of Sotolon is greatly dependent on O₂ concentration during aging and has a major impact on “age perception” in barrel stored Port wines.

Introduction

Port wine is a naturally sweet wine produced by interrupting alcoholic fermentation by the addition of grape spirit. These red wines, with an alcohol level of about 20% vol. and a few tenths of a percent of residual sugar undergo long periods of aging (>4 years), either for bottle aged ("Vintage" type) or barrel aged ("Tawny" and "Colheita" types) wines, up to 60 years and more. During this maturation period wine undergoes a number of compositional changes, the level of some substances decreasing over time while others increase or form. Changes become more pronounced with extended aging, significantly affecting the color and aroma of the wine.

The typical aroma developed during barrel storage, is the consequence of the chemical changes that take place during maturation, and is usually described as "maderised", "rancio" "burnt", "dry fruit", "nutty" and "spicy". The value of Port is related with the characteristic aroma developed during this long maturation. Aroma perception, is one of the most important parameters in food evaluation, the quality of barrel aged port wine, is evaluated basically on these sensorial properties. Moreover, the age certification of Port is also based on the sensory analyses performed by the "Instituto do Vinho do Porto" (IVP).

It has been suggested that the presence of aldehydes and methyl-ketones contribute to the "rancio" odor of barrel aged Port wine (2) and also in white wines (1). Other volatiles, were also studied in ports (3, 4, 5, 6, 7, 8). More recently, 3-hydroxy-4,5-dimethyl-2(5H)-furanone (Sotolon) has been suggested to be a key odorant in the aroma of aged port (9). This highly odor active molecule has been shown to contribute to the aroma of Jura wines "vin jaunes" (10, 13, 14, 15), "vins doux naturels" (14) Tokay wines (11), Botrytised wines (16) and Sherry wines (12).

The presence of Sotolon in foodstuffs has been reported to be the result of an aldol condensation between 2-ketobutyric acid and pyruvic acid (10), similar to the mechanism proposed for the 5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone formation in protein hydrolysates (17). The aldol condensation between acetaldehyde and 2-ketobutyric acid has also been suggested (11) to explain the presence of Sotolon in fortified type wines.

Other mechanisms, which exclude 2-ketobutyric acid as a reactant have also been reported, such as Sotolon formation from chemical or enzymatic conversion of 4-hydroxyisoleucine (18,19); as a product of reaction from hexoses and pentoses in the presence of cysteine (20); and from the aldol

condensation of hydroxyacetaldehyde and buta-2,3-dione (21). More recently (22) it has been demonstrated that Sotolon can be formed from ascorbic acid degradation products.

It is important to determine to what extent common industrial practices like SO₂ additions, pH corrections, and varying the type and volume of wine containers (and the associated variation in levels of dissolved oxygen) affect the rate of key odorant formation. Mathematical models can attempt to quantify the impact of a critical parameter on flavor formation. Kinetic studies are very important to know the extent of specific chemical reactions, the rate at which changes occur and to optimize food processing or storage conditions. The knowledge and control of kinetics parameters can greatly enhance the rate of generation of the desired flavor compounds (23).

Hence the aim of this work was to: (i) evaluate the impact of Sotolon on the “perceived age” of Port wine; (ii) determine which parameters (e.g. temperature, dissolved oxygen, pH and free SO₂ content) have the greatest effect on Sotolon concentration, (iii) and to establish mathematical models describing the rate of formation of Sotolon during storage, taking into account the critical parameters selected.

Material and Methods

Wine material: - Port Wine Samples.

Samples of “Colheita” type Port were aged in “pipas” (550 L spent-oak barrels) and those of “Vintage” type were aged in bottles until analysis. The samples were supplied by the Instituto do Vinho do Porto (IVP) after certification.

Wine Group 1 - Thirty-five samples of a single harvest ranging from 1 to 60 year-old “Colheitas” and seven samples of a single harvest ranging from 3 to 16 years-old “Vintage” types.

Wine Group 2 – “Forced Aging” experimental protocol: This protocol is similar to that of a previous study (8). Red port wine, 2 years-old (8L), with a pH=3.7, a dissolved oxygen content of 4.2 mg/L, an instantaneous potential of 222 mV and a free SO₂ level of 17 mg/L, was prepared in order to analyze the effect of parameters believed to be important in the aromatic modification of port wines, namely temperature, oxygen concentration, pH and SO₂ concentration (Figure 1).

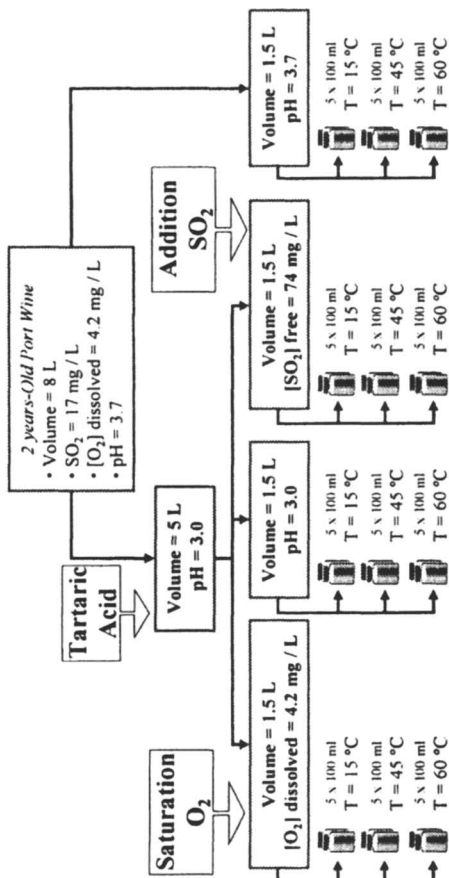


Figure 1. Schematic representation of the “forced aging” protocol.

To test the effect of pH, a Port wine sample was adjusted to pH 3 by adding tartaric acid. To test the effect of high concentrations of oxygen (oxidative environment), a portion of the pH 3.0 wine was saturated with oxygen (6.2 mg/L) by air bubbling. To test the effect of an exogenous antioxidant a portion of the pH 3 wine was adjusted to a free SO₂ level of 74 mg/L by adding potassium metabisulfite. The initial wine, having a pH=3.7, is referred to as pH 3.7 in the text. As shown in Figure 1, the 4 portions, each 1.5 liters, were divided into five sets of 100 mL and they were stored in sealed glass vessels. One part of each experiment was kept at 15°C, another one at 45°C and the last one at 60°C. The samples were analyzed at 0, 17, 32, 47 and 59 days of storage. The samples initially saturated with oxygen were re-saturated (7-8 mg/L) at each sampling stage. This experiment was performed in duplicate.

Wine Group 3 – Kinetics Studies: 3 liters of 2 year-old Port wine with a pH=3.6, a dissolved oxygen level of 1.2 mg/L, an instantaneous potential of 252 mV and a free SO₂ level of 22 mg/L, was divided in 2 portions; one was saturated with oxygen, and the other was kept free of oxygen. Each portion was further divided and stored in sealed vessels at 20, 30, 40, 50 and 60 °C for 77 days. Samples were analyzed at 0, 22, 30, 37 and 77 days. The samples initially saturated with oxygen were re-saturated (7-8 mg/L) at each sampling time.

Sensory studies

Sensory panel: The panel employed in all sensory measurements in this work was composed of eighteen persons; university students; Port winemakers and laboratory personnel. The panel is permanent and receives weekly training. Tests were performed in individual booths, using tulip glasses containing 30 mL of wine at room controlled temperature of 20 °C.

Ranking testing: The impact of Sotolon on the typical aroma of aged Port wine was evaluated using ranking tests carried out on wine samples of different ages with or without addition of Sotolon. Tests were performed in individual booths. A red light was used to mask visual differences between samples. The results were collected after three tasting sessions with a trained sensory panel of 18 assessors using the same sample preparation for each session.

Two wines certified by IVP, 4 year-old (“Ruby”) and 10 year-old (“10 anos”) were used. In order to obtain a “middle sample”, these two wines were blended, a mixture of 40% “Ruby” with 60% “10Anos” was chosen (“Blended-sample”-BS).

Three sets were studied, samples were supplemented with three different levels of Sotolon 25 µg/L; 50 µg/L and 100 µg/L. Each set had five samples, one with no addition, three supplemented samples and one sample of a different wine common with the next set as described in figure 2.

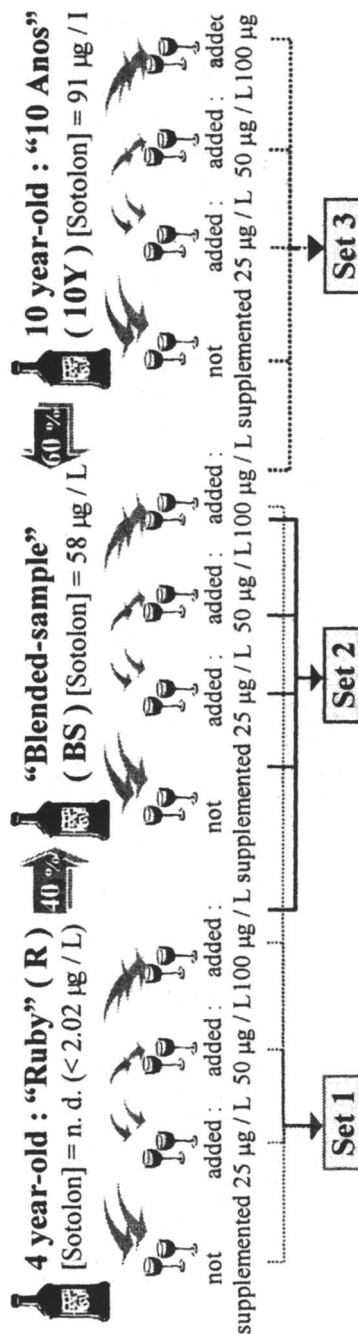


Figure 2. Sensory protocol: Wine sample preparation.

Samples were randomly coded using four alphanumeric characters. Each set was presented individually, on different days, to each assessor (three per session). The panelists were instructed to smell but not to taste and then order by age using a scale from 1 (youngest) to 5 (oldest) with unit intervals, rank repetition was not allowed. The correlation coefficients between the ranks for each assessor were calculated using the Spearman test (24). The ranks were converted to scores according to the method of Fisher and Yates (25). The sample ranked first of five was given a value of -1.16; the second -0.5; the third 0; fourth +0.5 and the fifth +1.16. The scores were then subjected to analysis of variance (ANOVA) in order to check if there was a significant difference among samples (at 5% level). To determine which samples were significantly different from another, Tukey's test was used. Samples were arranged according to magnitude, and the Honestly Significant Difference at 95% (HSD) was determined. Any two samples that differed by a value equal to or more than the HSD were regarded as significantly different (26).

Chemical Studies

Chemicals were obtained from Sigma-Aldrich, (Saint Quentin Fallavier, France): 3-hydroxy-4,5-dimethyl-2(5H)-furanone (Sotolon) (W36,340-5) (97%) and 3-octanol (21,840-5) (99%).

Hydroxy-4,5-dimethyl-2(5H)-furanone (Sotolon) quantification

Extraction procedure: To 50 mL of wine were added 50 μ L of 3-octanol (1/1 v/v hydroalcoholic solution) as internal standard and 5 g of anhydrous sodium sulfate to increase extraction efficiency. The 50 μ L of 3-octanol in 50 mL of wine yields, on a weight/volume basis, an internal standard concentration of 466 mg/L. The wine was extracted twice with 5 mL of dichloromethane (Merck, Spain). The two extracts were blended and dried over anhydrous sodium sulfate. Two mL of the extract was concentrated 5 times under a 1 mL/min nitrogen stream. Extracts were analyzed using a Varian CP-3800 gas chromatograph (USA) equipped with a Varian Saturn 2000 mass selective detector (USA) and a Saturn GC/MS workstation software version 5.51. The column used was STABILWAX-DA (60 m x 0.25 mm, 0.25 μ m) fused silica (Restek, USA). The injection port was heated to 220 $^{\circ}$ C. The split vent was opened after 30 sec. The carrier gas was Helium C-60 (Gasin, Portugal), at 1 mL/min, constant flow. The oven temperature was initially 40 $^{\circ}$ C (for 1 min), then increased at 2 $^{\circ}$ C/min to 220 $^{\circ}$ C and held at 220 $^{\circ}$ C for 30 min. All mass spectra were acquired in electron impact (EI) mode. The Ion Trap detector was set as follows: The transfer line, manifold and trap temperatures were respectively 230, 45 and 170 $^{\circ}$ C. The mass range was 33 to 350 m/z, with a scan rate of 6 scans/sec. The emission current was 50 μ A, and the electron

multiplier was set in relative mode to autotune procedure. The maximum ionization time was 25000 μsec , with an ionization storage level of 35 m/z . The injection volume was 1 μL and the analysis was performed in Full Scan mode. Detection and quantification thresholds were established in a Port wine diluted with 20% (v/v) aqueous-alcoholic solution and were found to be respectively 1.17 and 2.02 $\mu\text{g/L}$ (9).

Other analytical measurements: Redox potential, free SO_2 concentration and chromatic index were performed (27). Acetaldehyde, higher alcohols (28), acetals (7), 2-ketobutyric acid (9) and furanic aldehydes (29) were determined. The concentration of dissolved oxygen was measured using a “WTW 340 Oxygen Probe”.

Kinetic analysis: Statistical data analysis was performed using the Statistica program version 6.0 (30). The usual kinetic models reported in literature to describe kinetic of compound formation are zero order [$c = c_0 + kt$], first order [$c = c_0 \exp(kt)$] or second order [$1/c = 1/c_0 + kt$] reaction models. The Arrhenius equation $k = k_{\text{ref}} \exp(-E_a/R * (1/T - 1/T_{\text{ref}}))$ is usually applied to evaluate the effect of temperature on the reaction rate constant (31). For both levels of oxygen concentration a one step nonlinear regression method was performed and a regression analysis of the residuals was also carried out (32).

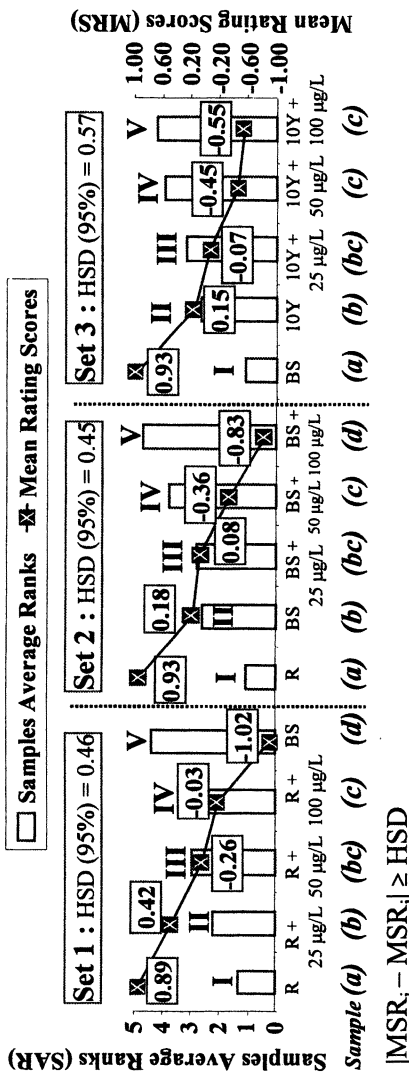
Results and Discussion

Sensory Studies

An estimation of the importance of Sotolon in establishing the character and persistence of aroma for “flor sherry” has already been established (12). Nevertheless, in order to gather more information concerning the impact of Sotolon on the typical aroma associated by the consumer with the age of Port wine, sensory analyses needed to be carried out.

Ranking results: A trained panel was given, three sets of wines supplemented with three levels of Sotolon as described in the material and methods section. The panel asked to rank the sample from youngest (1) to the oldest (5) based only on the perceived aroma.

The correlation between the rank order for age attributed by each assessor and the rank order for Sotolon concentration in samples (or real age) was calculated by the Spearman method (24). The ranks were converted into scores. and the ANOVA treatments, for 95% significant level, showed for each set no differences between assessors and differences among samples with a p-value for Sets 1 (4 years old), 2 (blended-sample) and 3 (10 years old) of p-value=1.233E-17; p-value= 1.489E-17 and p= 1.252E-11 respectively. Results are shown in figure 3.



(*) Samples not followed by the same letter are significantly different at 5 % level.

Figure 3. Samples evaluation results of the multiple comparison test (Significant level=95%).

To determine which samples differ significantly in “average ranked-age” after scoring translation, the comparison of the sample mean scores using HSD was calculated. Samples for the 3 sets were ranked by the panel on in increasing manner according to the real age and the increasing levels of Sotolon addition (Samples Average Rank). For Set_1 and Set_2 samples I, I', and V are significantly different from each other. The lower level of Sotolon addition in Set_1, was considered different from the non-supplemented sample (I / II). The distinction by the panel, between supplemented and non-supplemented samples was not verified in Set_2 (II / III). This fact can be related to the concentrations of Sotolon initially present in the non-supplemented samples, non-detected and 58 $\mu\text{g/L}$, respectively for “Ruby” and “Blended-Sample”, one below and the other above the odor threshold value in Port wine (19 $\mu\text{g/L}$) for this substance (1). In the second set it is important to note Sample V clearly rated as the oldest. Within Set_3 the differences are not so marked, nevertheless samples supplemented with 50 mg/L and 100 mg/L differed from the non-supplemented samples. These findings have already been reported in (9).

Chemical Studies

Sotolon Levels in Wines. The concentrations of 3-hydroxy-4,5-dimethyl-2(5H)-furanone (Sotolon) increases with storage time in barrels from a few tens $\mu\text{g/L}$ in young wines, to about 100 $\mu\text{g/L}$ in 10 year-old wines and reaching 200 $\mu\text{g/L}$ after 10 additional oxidative aging years. The highest Sotolon contents were observed for wines older than 50 years, *i.e.* almost 1 mg/L (figure 4). The same kind of behavior was also observed for bottle-aged wines the “Vintage” type (Figure 4). For both types a high correlation coefficient ($r > 0.90$) clearly demonstrates the dependence of Sotolon content on the storage time.

Although fewer samples of “Vintage” wines were analyzed, it appears clearly that for the same period of aging (16 years), the rate of formation of Sotolon is substantially higher, close to 10-fold higher in “Colheitas”. The levels of dissolved oxygen seem to play a determining role in the kinetics of Sotolon formation. In fact, the typical values of dissolved oxygen measured in barrels in Port wines cellars were close to 2-4 mg/L . It has been extensively reported that this concentration in barrels is inferior to 0.5 mg/L in bottles. It is also important to note, that the descriptors associated with bottle-aged port wines are related with “floral” and “fruit-like” a “younger” aroma character, when compared with the typical notes of oxidative aging. Considering uniquely the single effect of Sotolon in Port wine expressed by the “Odor Aroma Value”

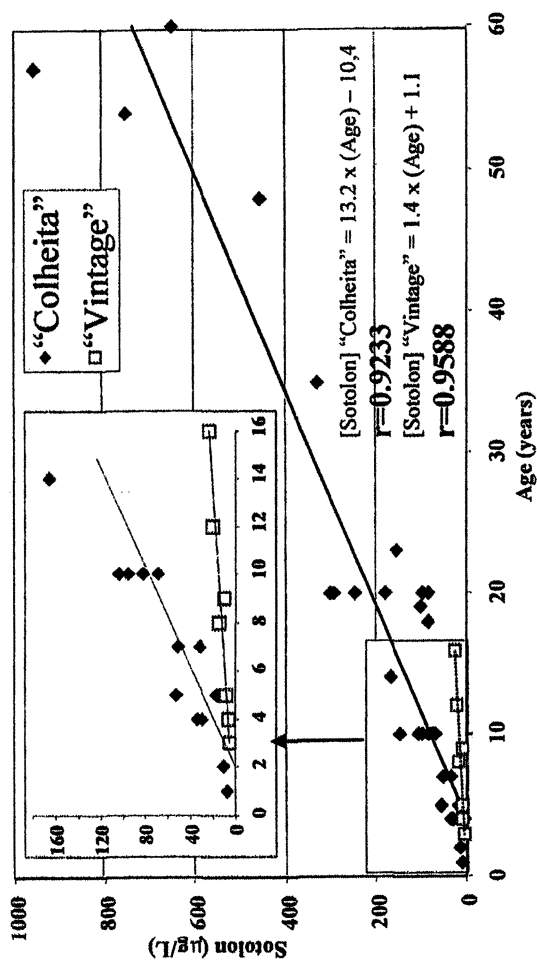


Figure 4. Concentration of Sotolon in "Colheita" and "Vintage" Ports ($\mu\text{g/L}$).

[OAV = (odor threshold / concentration)_{matrix}] this substance has a positive impact on aroma of barrel stored wines close to 10 years-old (OAV > 1) while in bottle-aged Ports this is only observed after 20 years or more. These results are in close agreement with the empirical observation in the Port wine industry upon which the “rancio” aroma constitutes a “quality factor” for wines aged in barrels for more than 10 years, while it is not considered a major attribute of the total aroma of “Vintage” types.

Sotolon Formation: Among the mechanisms reported in literature concerning 3-hydroxy-4,5-dimethyl-2(5H)-furanone formation, two seem to be of particular interest in order to explain the formation of this molecule in Port wine. Both involve a common step, the aldol condensation between two carbonyl compounds followed, by cyclization. In “vin jaune” Sotolon could be formed during aging by the aldol condensation of acetaldehyde and 2-oxobutyric acid produced from threonine by an enzymatic reaction, only possible due to “flor”, i.e. the presence of a yeast belonging to the *Saccharomyces* genus (13). In Port wine, this “flor” is absent; therefore, there is no microbial intervention after the end of alcoholic fermentation, with the exception of some contamination due to alcohol resistant lactic bacteria (33). However, a strictly chemical degradation of threonine into 2-ketobutyric acid under acidic conditions, has also been suggested (34) (Figure 5).

The presence of 2-ketobutyric acid in Port wine was investigated (9). Although the quantities found could reach 2 mg/L, these were always lower than 0.5 mg/L for wines older than 10 years. No correlation between the levels of keto-acid neither with age nor with the amount of furanone could be found. Conversely a very high correlation was observed with acetaldehyde ($r=0.8906$). Thus, 2-ketobutyric acid may contribute to Sotolon formation, but taking into account the linear trend observed with time for the Sotolon levels, it seems unlikely that this keto-acid constitutes the only source of Sotolon in Port wine.

In addition, other work showed that 3-hydroxy-4,5-dimethyl-2(5H)-furanone can be formed thanks to a Maillard reaction of hexoses and pentoses in the presence of cysteine (20). Due to the non-linear structure of Sotolon, its formation cannot simply be explained directly from sugar cyclization during the Maillard reaction, like other furanones such as Furaneol. Hence, it is likely that Sotolon results from rearrangement of Amadori products of low molecular weight like butan-2,3-dione (diacetyl) and hydroxyacetaldehyde, via an aldol condensation (Figure 6).

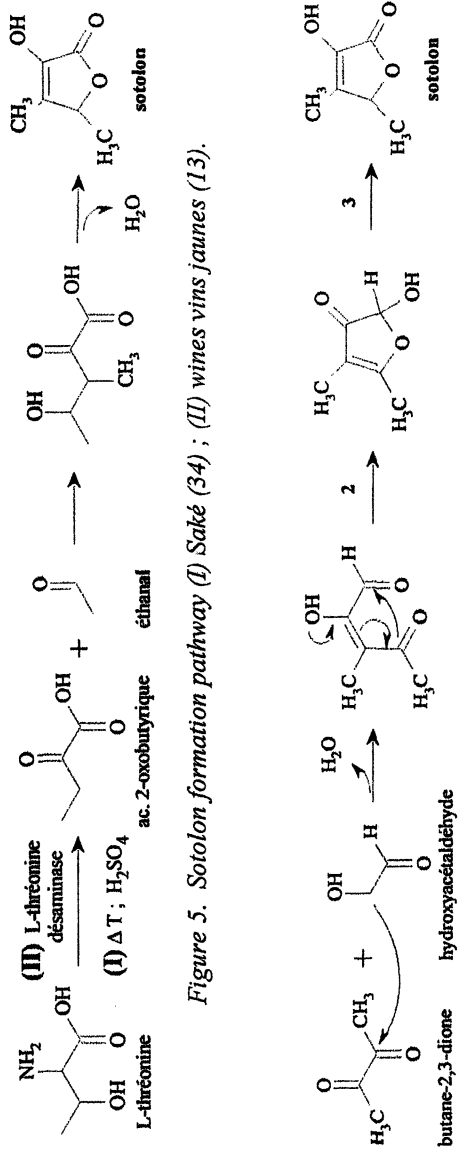


Figure 5. Sotalon formation pathway (I) Saké (34) ; (II) wines vins jaunes (13).

Figure 6. Mechanisms of Sotalon formation by Maillard reaction (21).

Although the presence of hydroxyacetaldehyde needs to be confirmed, diacetyl levels in Ports can reach almost 10 mg/L. Taking into account the high level of sugar in Port wine (approximately 100 g/L) and the high correlation coefficient of furanic aldehyde sugar degradation products, like furfural, 5-methylfurfural and in particular 5-hydroxymethylfurfural ($r=0,9015$), with Sotolon, this last mechanism seems to fit the observed behavior of the continuous formation of Sotolon during aging.

Forced Aging Experiment : On an empirical basis, Sotolon appearance seems to be closely related with oxygen levels during storage. For this reason, an experimental protocol was established in order to evaluate the effect of some parameters which are believed to be important in the aromatic modification of port wines, namely temperature, oxygen concentration, pH and SO₂ concentration.

As shown in Figure 7, the formation of Sotolon is clearly related to temperature and to the presence of oxygen in the wine

In fact, none of the treatments at 15 °C led to significant changes in the concentration of Sotolon. On the other hand, at 45 °C there was a considerable increase in Sotolon amount with time, except for the samples treated with SO₂. The same behavior was observed for samples stored at 60 °C, with particular emphasis for the samples treated with oxygen. In fact, the large amounts of Sotolon found in this set are noteworthy: After 59 days of storage at 60 °C, the concentration of Sotolon reached 300 µg/L, for a total of 22 mg/L consumed oxygen. The relative rate of formation (*RRF*) of this substance was estimated in a rough manner by considering that the concentration changes linearly with time. The ratio of the respective slopes was calculated and the results show that the rate of formation observed for this substance is 10 times higher for O₂ treated samples than for pH 3.0 (non- O₂ supplemented) samples.

Conversely, we found that SO₂ treatment “blocks” furanone formation. These results are in agreement with those published previously (35). This observation can be explained by (i) the consumption of dissolved oxygen due to the high reactivity of this species with sulfur dioxide and (ii) the combination of SO₂ with the carbonyl group of the precursors consequently blocking the aldol reaction.

The amounts in Sotolon are mainly dependent upon, in decreasing order of importance, the temperature and the amount of dissolved oxygen. SO₂ plays an important role as a “inhibitor” on furanone formation.

Kinetics Studies : After determining the parameter with the highest effect upon Sotolon concentration in port wines, attempts were made to establish a mathematical model, which reflects the impact of oxygen level, temperature as well as the time of storage, on the rate of formation of Sotolon.

To determine an unambiguous kinetic model for a certain reaction it is important to know the reaction stoichiometry and mechanism. However, as explained above, the formation of Sotolon is complex, involving incompletely

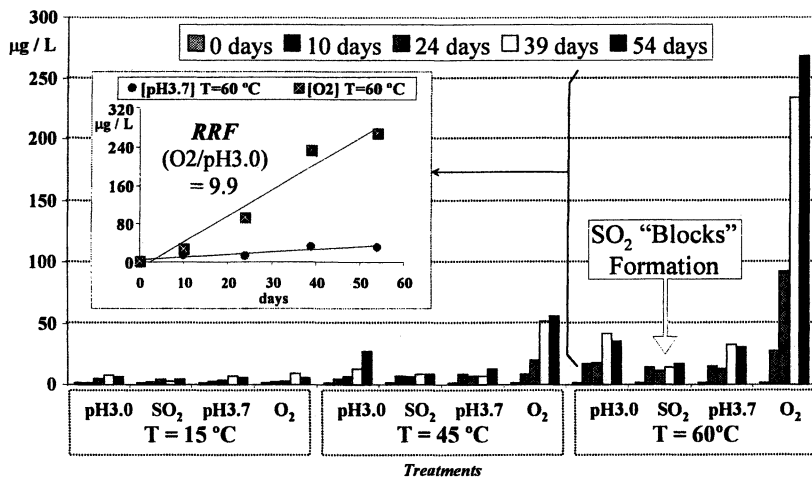


Figure 7. 3-hydroxy-4,5-dimethyl-2(5H)-furanone (Sotolon), concentrations in samples of a "forced aging" experiment and the relative rate of formation (RRF) for treatments O₂ / pH3.0 at T = 60 °C.

known mechanisms. Therefore, considering the total reaction mechanism, only an apparent reaction rate can be calculated and the resultant kinetics determined (36). As a result of experimental data modeling, formation of Sotolon, both in the saturated and non- O₂ supplemented experiments followed first order reaction kinetics. Also, for both cases, the temperature dependence of the reaction rate constant was well described by the Arrhenius equation (see Figures 8A, 8B), respectively for the saturated and non- O₂ supplemented experiments).

Statistical analysis using a one step non-linear regression method was applied in order to estimate kinetic parameters. Activation energies and rate constants estimated at the reference temperature of 40°C and corresponding 95% confidence intervals are reported in Table I.

For O₂ saturated samples, the Sotolon concentration increases exponentially with temperature, illustrating the high sensitivity for this parameter. This fact is in agreement with the higher Arrhenius activation

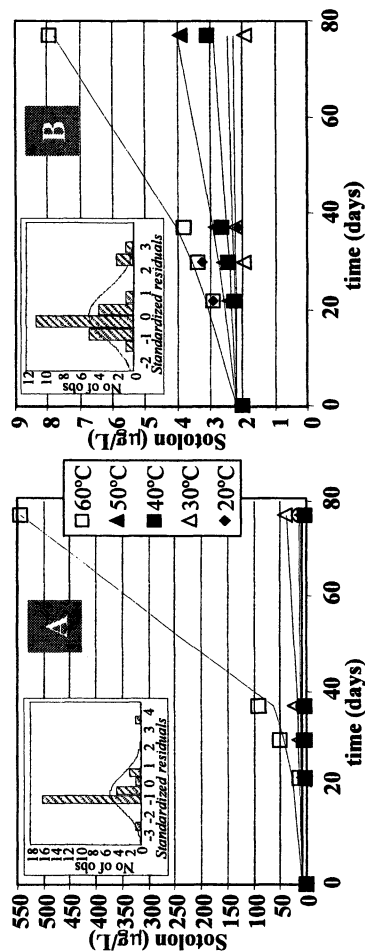


Figure 8. One step non-linear regression modeling for Sotolon formation for A-saturation regimes and B-not supplemented samples.

Table I. Kinetic parameters for the formation of Sotolon, using the one-step nonlinear regression method, for oxygen saturated and non-O₂ supplemented samples.

	$c = c_0 \exp\left(-k_{ref} \times \exp\left(\frac{E_a}{R} \left(\frac{1}{T} - \frac{1}{T_{ref}}\right)\right)\right)$	model	E _a (kJ/mol)	k _{ref} (days ⁻¹)	C ₀ (µg/L)
[O ₂] dissolved = Saturation	first	first	92.0 ± 30.7	0.0065 ± 0.0049	8.44 ± 2.56
[O ₂] dissolved = Not Supplemented	first	first	66.2 ± 24.8	0.0036 ± 0.0022	2.20 ± 0.20

energy found for the O₂ saturated experiment. The total consumed oxygen was for this set 30 mg/L. On the other hand, without the presence of oxygen only a very slight increase of Sotolon formation for samples stored at 60 °C was observed. This result is translated by the smaller reaction rate constant found for the non-supplemented experiment.

Although predictions are out of the scope of this modeling work, due to constraints of the experimental design, we have attempted to estimate Sotolon concentration of a 16 year old Port wine, for a temperature of 17°C, using both models for the two oxygen levels. This was done using real samples originating from the same wine lot, but stored in barrels (high O₂ level) and bottles (low O₂ level). The observed concentrations were respectively 90 µg/L and 23 µg/L, surprisingly, similar to the predicted values using both models for the two oxygen levels, respectively, 83 µg/L (high O₂) and 35 µg/L (low O₂). In order to use these or other mathematical models as predictive tools it seems clear that oxygen levels need to be taken into account.

Conclusion

Sotolon (3-hydroxy-4,5-dimethyl-2(5H)-furanone) concentration in Port correlates positively with years of barrel storage aging. Within certain limits Sotolon concentration can also correlate with the intensity of typical aroma descriptors “nutty”, “spicy” and the “perceived age” of barrel stored Port wine. This phenomenon can be demonstrated even with Sotolon additions to unaged base port.

The quantification of Sotolon both in “Colheita” and “Vintage” types clearly shows a high dependence between Sotolon level, maturation time and dissolved oxygen.

The Sotolon formation was studied as a function of temperature, pH, SO₂ and oxygen concentration. Temperature and to a lesser extent, dissolved oxygen were the most important parameters influencing the rate of Sotolon formation. A first order model was applied and the Arrhenius equation described well the temperature dependence of the reaction rate constant. Therefore, oxygen levels during aging have a major impact on “age perception” in Colheita” and “Vintage” Port wine.

Considering the key role played by Sotolon on the sensory “quality” of barrel aged Port wine, these findings seem promising in order to provide to the industry a mechanism of stock management, in particular for blended, “Tawny” Port, wines in order to modulate the “technological age” of Ports.

References

1. Cantarelli C.; Etude de la madérisation et de sa prévention dans les vins blancs. *Deuxième Symposium Inst. d'Oenologie. Bordeaux Cognac*, 2, 1967, 392-407.
2. A C. Silva Ferreira, Bertrand A; Evolution de quelques constituants du vin de Porto au cours du vieillissement. Etude particulière de composés carbonylés. In *Oenologie 95, 5eme Symposium International d'Oenologie*; Lonvaud-Funel, A, Ed. Lavoisier Tec & Doc: Paris, 1996 ; pp 419-423.
3. Simpson, R. *J. Sci. Food Agric.* **1980**, 31, 214-222.
4. Williams, A.A.; Lewis, M.L.; May, H.V. *J. Sci. Food Agric.*, **1983**, 34, 311-319.
5. V. A. P. Freitas, Paula Ramalho, Zélia Azevedo and Adelina Machado. *J. Agric. Food Chem.* **1999**, 47, 4327-4331.
6. Rogerson, F; H., Castro, N., Fortunato, Z., Azevedo, A, Macedo and Freitas, V. *J. Agric. Food Chem.* **2001**, 49, 263-269.
7. Silva Ferreira, A.C.; Barbe, J.C.; Bertrand, A. *J. Agric. Food Chem.* **2002**, 50(9) 2560-2564.
8. Silva Ferreira, Rodrigues, P., Hogg, T.; Guedes de Pinho, P. *J. Agric. Food Chem.* **2003**, 51(3) 727-732.
9. Silva Ferreira, A.C.; Barbe, J.C.; Bertrand, A. *J. Agric. Food Chem.*, **2003**, 51(3) 4356-4363.
10. Dubois P., Rigaud J. and Dekimpe J. *Lebensm. Wiss. Technol.* **1976**, 9, 366-68.
11. Martin B. and Etievant, P., X. *HRC & CC* **1991**, 14, 133-5.
12. Martin, B.; Etiévant, P. X.; Le Quéré, J. L.; Schlich, P. *J. Agric. Food Chem.*, **1992**, 40, 475-78.
13. Pham, T. T.; Guichard, E.; Pascal, S.; Charpentier, C. *J. Agric. Food Chem.*, **1995**, 43, 2616-2619.
14. Shneider, R., Baumes, R.; Bayonove, C; Razungles, A. *J. Agric. Food Chem.* **1998**, 46, 3230-3237.
15. Guichard E., Pham T. T. and Etievant P. X. *Chromatographia*, **1993**, 37(9-10), 539-42.
16. Masuda M., Okawa E., Nishimura K. and Yunome H.; *Agric. Biol. Chem.*, **1984**, 48, 2707-10.
17. Sulser H., Depizzol J. and Buchi W. *T. Food Sci.* **1967**, 32, 611-15.
18. Lerk K. and Ambuhl M., Biotechnological production of 4,5-diméthyl-3-hydroxy-2(5H)-furanone. in: *Bioflavor 95. Analysis-Precursor Studies-*

- Biotechnology*; Etiévant, P.; Schreier, P., Eds.; Les Colloques no.75, INRA: Paris, 1995, p.p.: 381-84.
19. Blank I., Lin J., Fumeaux R., Welti D. H. and Fay L. B. *J. Agric. Food Chem.*, **1996**, *44*, 1851-56.
 20. Hofmann, T; Schieberle, P. *J. Agric. Food Chem.*, **1997**, *45*, 898-906.
 21. Hofman T. and Schieberle P.; Identification of the key odorants in processed ribose-cysteine Maillard mixtures by instrumental analysis and sensory studies. *Spec. Publ. - R. Soc. Chem.*, 1996, 197 (Flavour Science), 175-81.
 22. T. Konig, B. Gustche, M. Hartl, R. Hubsher, P. Schereier and W. Schwab *J. Agric. Food Chem.*, **1999**, *47*, 3288-3291.
 23. Yaylayan, V. and Forage, N.G. *Journal of Agricultural and Food Chemistry* **1991**, *39*, 2, 364-369.
 24. Saporta G.; *Probabilités analyse des données et statistique*, Ed., Technip-Paris, 1990.
 25. Laboratory Methods for Sensory Evaluation of Food, publication 1637, Communications Branch, Agriculture Canada, Ottawa K1a 0C7, ISBN: 0-662-01271-2, 1982.
 26. Morten Meilgaard, Gail Vance Civille, B. Thomas Carr. *Sensory Evaluation Techniques* 3rd eds. 1999, CRC Press LLC.
 27. Ribereau-Gayon, J.; Peynaud, E.; Ribereau-Gayon, P. and Sudraud, P. *Traité d'Oenologie. Sciences et Techniques du Vin.*, Tome 1. Analyse et contrôle des vins. 1976, Dunod eds., Paris.
 28. Bertrand, A.. *Colloque Soc. Fr. Microbiol.*, Reims, **1981**, 251-67.
 29. Barbe, J.-C.; Silva Ferreira, A.C.; Bertrand A. *Oenologie 1999 6^{ème} Symposium International d'Oenologie*. Tec et Doc Ed., Paris, France, 2000, pp. 497-500.
 30. Statistica Inc. (2001). *Statistica Software: Release 6.0*, Oklahoma, USA.
 31. Van Boekel, M.A.J.S. *Journal of Food Science* **1996**, *61*, 3, 477-485, 489.
 32. Arabshahi, A.; Lund, D.B. *J. Food Process Eng.*, **1985**, *7*, 239-251.
 33. Couto, J. A. and Hogg, T. *Journal of Applied Bacteriology* **1994**, *76*, 487-491.
 34. Takahashi, K.; Tadenuma, M.; Sato, S. *Agric. Biol. Chem.* **1976**, *40*, 325-330
 35. Martins, S. I. F. S. *Unravelling the Maillard reaction network by multiresponse kinetic modelling*. Ph.D. Thesis, Wageningen University, The Netherlands, 2003.
 36. Levenspiel, O., *Kinetics of homogeneous reactions*. In *Chemical reaction engineering*, 3rd ed.; John Wiley & Sons: New York, 1999; pp 13-32, chapter 2.

Chapter 11

Role of Polymethoxylated Flavones in Citrus Flavor

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Introduction

Citrus is the world's most consumed fruit and is valued as a food flavor by all major cultural groups. The immense popularity of citrus flavor is evidenced by the fact that orange is one of the top flavors sold world-wide by the commercial flavor industry. In addition to desirable flavor, citrus products are also considered healthy to consume. Recent interest in health promoting components other than Vitamin C has focused attention on the citrus flavonoids. While this recent interest may seem new, research into citrus flavonoids has been underway for some time. In the 1930's, Rusznyak et al. proposed that citrus flavonoids should be referred to as a new vitamin – Vitamin P (1). By the 1950's, citrus flavonoid mixtures were routinely available as a byproduct of the juice concentration business and commercialization ideas were being suggested (2). On a weight basis, the dominant flavonoids in citrus are flavanone glycosides like hesperidin in orange or naringin in grapefruit. Most flavor and health research involving citrus flavonoids had focused on these molecules. More recently, interest has focused on other citrus flavonoids such as polymethoxylated flavones (PMFs).

PMFs are based upon the C₆-C₃-C₆ flavone structure shown in Figure 1. The double bond in the 2-3 position and the oxygen attached at the 4 position characterizes the flavone structure as compared to other flavanoids such as the flavanones which are also common in citrus. Plant flavanoids often occur with a sugar group attached to the flavonoid backbone, the entire molecule is then called a glycoside. The most common citrus flavonoids – hesperidin and naringin – are glycosides. PMFs are aglycones with varying numbers of methoxy groups attached to both A and B rings. Due to the lack of a sugar moiety, these compounds have relatively poor water solubility. While hesperidin and naringin are found at levels of hundreds of ppm, the concentrations of PMFs found in citrus juices are much lower (typically 2 to 20 ppm). PMF's are found in few foods other than citrus, making citrus the dominant source of PMF's in the typical human diet.

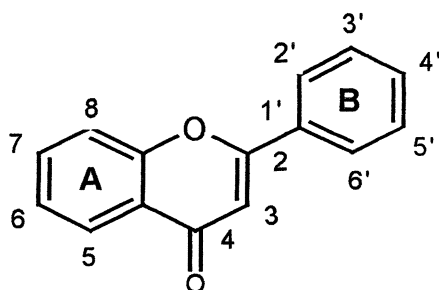


Figure 1. The flavone structure

The highest levels of PMFs in citrus are found in the orange and mandarin/tangerine cultivars, with much smaller levels in grapefruit and other citrus. The major PMF compounds in citrus, along with abbreviations for each that will be used in the rest of this paper are listed in Table I. The major PMF compounds in citrus contain from 4 to 7 methoxy groups. Relatively non-volatile, the molecular weights of these PMFs range from 342 to 432. In pure form, the compounds are colorless solids at room temperature. To the non-specialist, the best known compounds are nobiletin (5,6,7,8,3',4'-hexamethoxyflavone) and tangeretin (5,6,7,8,4'-pentamethoxyflavone).

Research into the health benefits of the PMFs has been extensive; two recent reviews are given in references (3) and (4). PMF effects on cancerous tumor growth/prevention (4), cholesterol metabolism (3), and inflammation (4) have been studied in the most detail. Recent studies have also looked at the effect on insulin resistance (5) and treatment of brain disorders (6).

Additionally, toxicity studies indicate that the PMFs (and their metabolites) have very low toxicity in mammals (7).

Table I. Major citrus PMF compounds

abbreviation	common name	chemical name
SIN	sinensetin	5,6,7,3',4' - pentamethoxyflavone
HMQ	hexamethoxyquercetagenin	3,5,6,7,3',4' - hexamethoxyflavone
NOB	nobiletin	5,6,7,8,3',4' - hexamethoxyflavone
TMS	tetra-O-methylscutellarein	5,6,7,4' - tetramethoxyflavone
HMF	heptamethoxyflavone	3,5,6,7,8,3',4' - heptamethoxyflavone
TAN	tangeretin	5,6,7,8,4' - pentamethoxyflavone

Beyond the health benefits, we were motivated to investigate the role of PMFs on the citrus flavor profile. Although citrus flavor is usually associated with the more volatile terpenes, esters and aldehydes, it is well known that citrus flavors based upon volatile fractions can not entirely reproduce the flavor profile of citrus juices. We were interested to determine the role that PMFs played in the citrus flavor profile.

Analysis

A relatively simple HPLC method was developed to analyze the PMF content in citrus products using a UV detector. Figure 2 shows the absorption spectrum for TAN, NOB, and HMF between 200 and 400 nm. The PMFs are all good absorbers, characterized by strong absorption at low wavelengths as well as a second absorption peak near 320 nm. Using the second absorption peak for PMF detection substantially reduces interference from other compounds.

The method was run on an Agilent 1100 HPLC using a diode array detector and a reverse phase C18 column (Agilent Zorbax SB-C18 Stablebond 4.6 mm x 250 mm; 5 μ m particle size). A mobile phase consisting of 50% acetonitrile : 50% water (acidified to pH 2.1 with phosphoric acid) with isocratic flow at 1 mL/min provided adequate separation of the typical PMF compounds. Figure 3 shows a typical absorbance spectrum in the wavelength range 300 to 330 nm for PMFs extracted from orange juice. The PMFs all elute within 15 minutes with fairly good peak resolution. This method provides similar results to that of Ooghe et al. (8) but with a simpler solvent system.

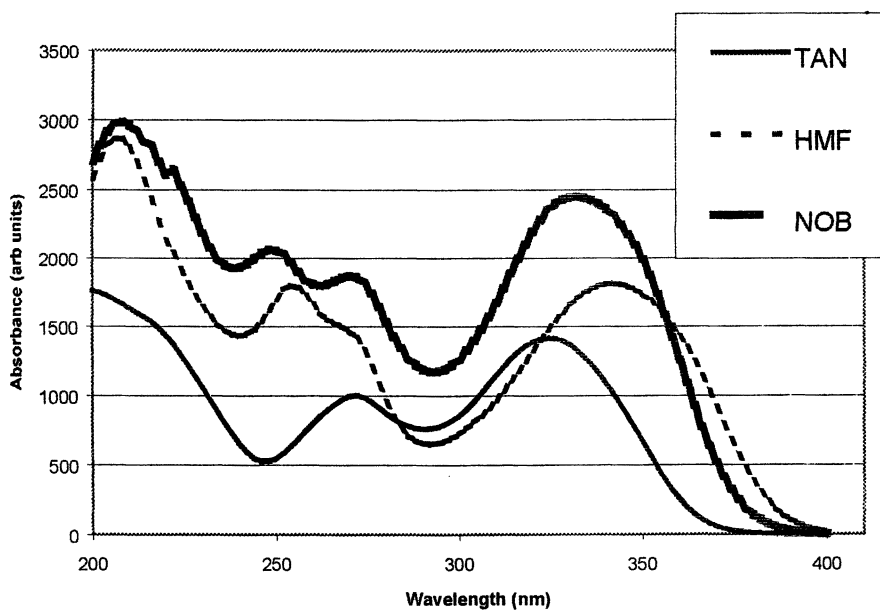


Figure 2. UV absorbance spectrum for TAN, HMF and NOB.

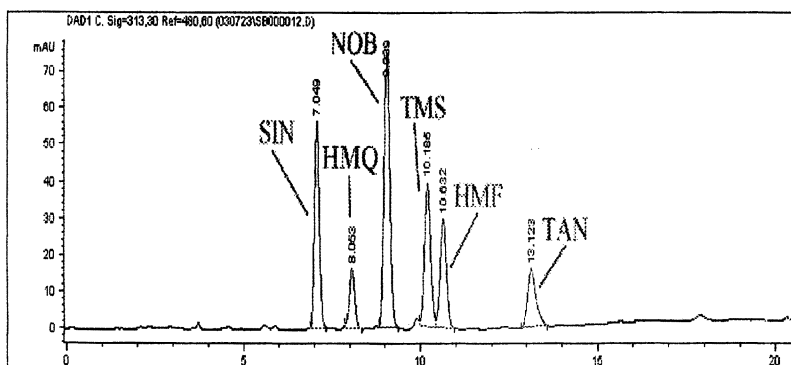


Figure 3. Typical HPLC chromatogram for orange juice.

Sample preparation before HPLC analysis differs for juice and peel oil samples. Juice samples were extracted 1:1 on a weight basis with solvent (10% w/w dichloromethane + 90% hexane) (40 g juice + 40 g solvent), the solvent was separated and evaporated to 0.5 g using a Turbovap II model concentration. This concentrated mixture was injected into the HPLC. Extraction recovery for the PMFs was determined to be 89 %. Oil samples were diluted to 10% (w/w) in ethanol and injected directly. In both cases, injection volumes of 5 μ L were used. All oil samples in this study were winterized for at least 30 days at a temperature of 4 C or less.

Calibration standards of >90% purity were obtained for SIN, NOB, TMS, HMF and TAN (9). The standards were used to prepare an external calibration for the components. No standard was available for HMQ, which is a relatively minor peak in the citrus PMF spectrum, so the SIN calibration was used for this peak. The relative uncertainty of the method, based upon multiple injections of the same samples, is less than +/- 5%. We estimate the absolute uncertainty of the calibration to be within +/- 20% based on the method reproducibility and the high purity of the PMF standards.

Natural Occurrence

Eleven commercial samples of orange juice (OJ) and one commercial sample of tangerine juice (TJ) were analyzed to determine typical levels of total PMF content. These results are shown in Figure 4. The total PMF content of the OJ samples ranges from 1.5 ppm to 8.5 ppm with an average of 5.0 ppm and standard deviation of 2.0 ppm. The reason for the relatively broad spread in PMF content is unknown. It may be due to the different combinations of orange varieties used in the commercial samples, or perhaps how the oranges were processed. The tangerine juice sample showed the highest PMF content at 9.5 ppm. TJ has traditionally been associated with higher PMF content, but the level we found was not substantially above the level found in the highest OJ sample. Because tangerine juice consumption is so small, we were not able to obtain additional commercial samples to analyze. Pupin et al. (10) found comparable results on PMF content in OJ in a study on Brazilian orange juice. He also found substantial variability in the juice PMF content due to the processing method used for juice extraction.

Figure 5 shows a comparison of the measured PMF levels in tangerine and orange peel oil. The same PMF compounds are found in both oils, but the relative proportions are different. For example, TAN comprises a larger

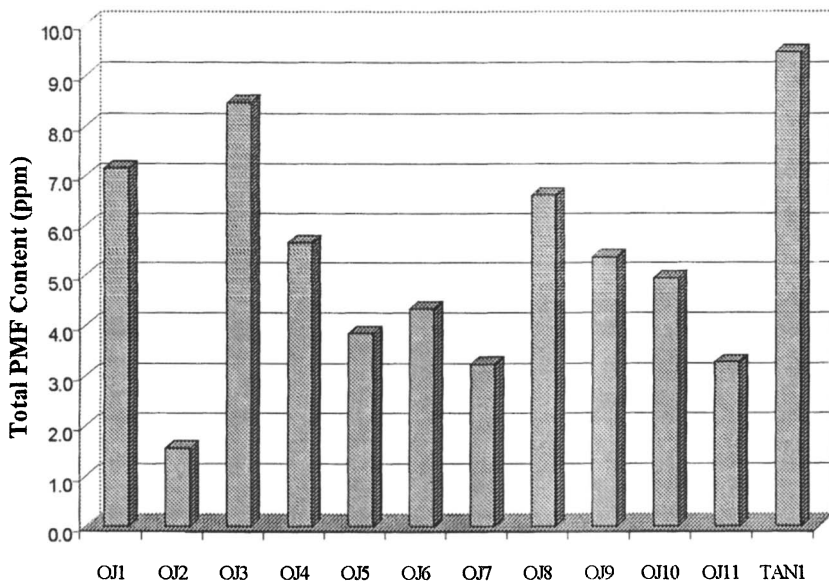


Figure 4. Total PMF content of commercial orange and tangerine juices

percentage of the total PMF content in tangerine oil than orange oil. Based upon the analysis of 12 orange oil samples and 33 tangerine oil samples, we find substantial variation in the total PMF content. In general, the typical level of total PMFs in tangerine oil is between 4500 and 10,000 ppm (average 7700 ppm, standard deviation 3100 ppm) while the concentration in orange oils falls between 3500 and 5200 ppm (average 4140 ppm, standard deviation 460 ppm). While verifying that tangerine oil is a more concentrated source of PMFs than orange oil, the reason for the substantial variation in concentration between samples was unknown. Our results are similar to those by Gaydou et al. (11) who reported PMF content for a number of different orange and mandarin varieties. Gaydou found that among 7 orange varieties, the peel oil PMF content averaged 2840 ppm (standard deviation 760 ppm) and among 8 mandarin varieties the average peel oil PMF content was 4400 ppm (standard deviation 1200 ppm).

In order to explore the influence of citrus variety on PMF content, we analyzed 33 different tangerine peel oil samples most of which were collected during variety-specific processing. The results are shown in Table II. Most of the samples originated from tangerine varieties grown in Florida. The Mexican tangerine oil samples represented commercial oil samples from Mexico. They

are likely dominated by the Dancy variety, but the varietal purity cannot be verified. The Chinese tangerine samples were commercial oil samples obtained from China. A number of different tangerine varieties are grown in China and our samples contain an unknown mixture of varieties. The Chinese and Mexican oil samples are shown for comparison purposes only. The Orlando tangelo, which is a tangerine hybrid, results from a cross between a Dancy tangerine and Duncan grapefruit. The average PMF content by variety varies more than a factor of two from 4500 ppm to 11,900 ppm. However, the inter-variety data also shows large variation with many relative standard deviations above 20%. Furthermore, US Dancy oil seems substantially different from Mexican oil which is also likely from Dancy tangerines. While variety is likely an important contributor to PMF variability, it is not possible to rule out other factors such as fruit maturity, growing conditions, and processing conditions. The relatively small sample-set analyzed here would need to be expanded substantially to address these questions. However, it is clear from the data that large sample-to-sample variation is not unusual.

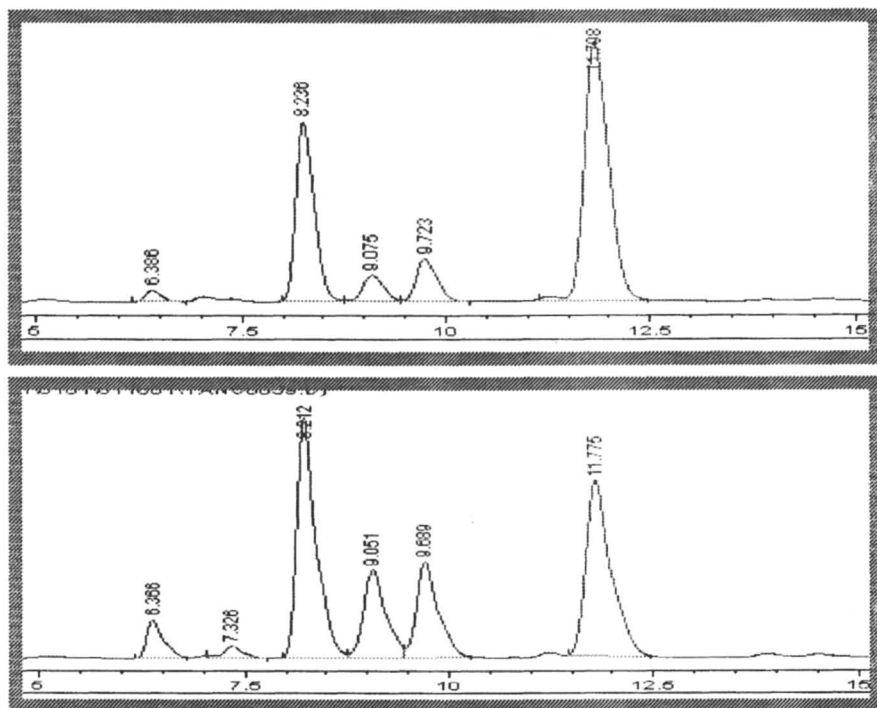


Figure 5. HPLC chromatograms for typical tangerine peel oil (top) and orange peel oil (bottom) samples. Peak IDs: retention time 6.3 = SIN, 7.3 = HMQ, 8.2 = NOB, 9.0 = TMS, 9.6 = HMF, 11.7 = TAN.

Table II. PMF content of various tangerine varieties

Varietal	avg. total PMF (ppm)	std dev (%)	# samples
Orlando Tangelo	11900	9	4
Sunburst	9300	28	6
Dancy (US)	8600	27	7
Mexican	4900	16	4
Chinese	4700	21	7
Honey	9600		2
Robinson	5900		3

A third byproduct from citrus processing is citrus molasses (CM). CM is obtained from the spent citrus peel after the juice and peel oils are extracted. The peels are further processed to squeeze out residual juice sugars and peel oils. This mixture is concentrated by distillation which removes water and many of the volatile oil components present. A sample of 33 Brix orange molasses was analyzed for PMF content to compare with our other results. The HPLC spectrum (Figure 6) shows a very different absorption spectrum from the typical juice sample. A large (unidentified) peak is seen eluting before 2.5 minutes with the PMF peaks eluting later. The total PMF content in this

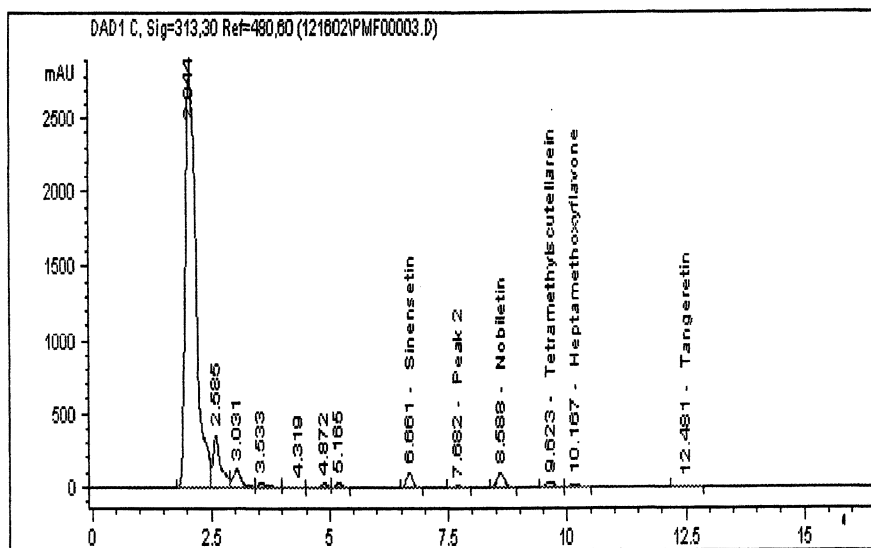


Figure 6. HPLC chromatogram for orange peel molasses sample (Peak 2 = HMQ).

molasses sample was 300 ppm. Our result is similar to the values reported in CM in an earlier study by Manthey et al. (12).

PMF Byproduct

A commercial proprietary process has been developed to isolate citrus PMFs at concentrations between 60 – 70% using commercial-scale separation techniques. The PMF fraction is isolated from orange/tangerine citrus oils and the PMF distribution closely matches the natural distribution in the raw materials. Figure 7 shows an HPLC chromatogram for a typical sample. Because only physical separation techniques are used in the manufacturing, the PMF fraction remains 100% natural and suitable for FTNF (From The Named Fruit) applications where orange and tangerine juices are used. The non-PMF content of the commercial PMF fraction is largely uncharacterized. However, the fraction is deeply-colored reddish-orange so it likely contains carotenoids originally present in the peel.

During initial flavor studies of the PMF compounds, it was discovered that they had very little flavor character that a non-expert would associate with citrus. However, an interesting effect on the apparent mouthfeel of PMF-containing beverages was identified. The contribution to mouthfeel from the commercial PMF fraction was evaluated by means of a flavor panel. Two types of tests were used. In the first case, an expert citrus flavor panel was presented with samples of the PMF fraction dosed in a sugar-acid taste solution (10 Brix, 0.2% citric acid) at two doses – 10 ppm and 20 ppm. Panelists were asked to qualitatively compare the taste in solutions with and without added PMF. The results are shown in Table III. The PMF was perceived to contribute an overall “mouthcoating” or “thickening” perception to the taste solution. The “thickening” comments referred to the panelists’ perception only as there was no measurable change in the beverage viscosity with the addition of the PMF fraction at these low levels. No panelists associated a strong citrus flavor to the PMF fraction. As the concentration of the PMFs was increased in a beverage, panelists reported an increasing perception of bitterness.

Table III. Taste panel results - PMF fraction contribution to mouthfeel

Dose (ppm)	taste panel comments
10 ppm	“Very weak citrus character, noticeable increase to apparent thickness and mouthcoating effect”
20 ppm	“Slight citrus character, noticeable mouthfeel improvement, slightly bitter and/or astringent”

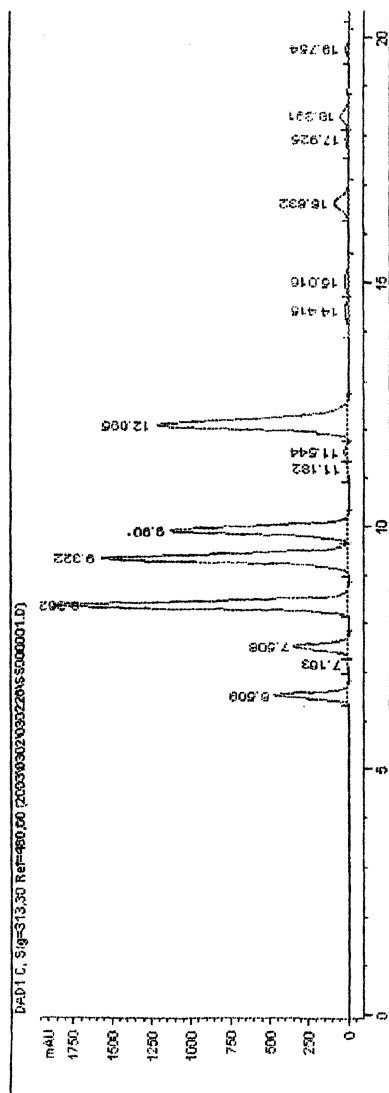


Figure 7. HPLC chromatogram for commercial PMF fraction. Peak IDs: retention time 6.5 = SIN, 7.5 = HMQ, 8.3 = NOB, 9.3 = TMS, 9.9 = HMF, 12.0 = TAN.

Since the mouthfeel effect was associated by the panelists with the typical mouthfeel of citrus juices, a second taste test was performed to better study this effect. In this second test, the expert panel was presented with four samples of an orange-flavored beverage. The first sample was prepared using 2% orange juice in the beverage formulation as well as sugar, acid and a volatile-based orange flavor. This sample also included the commercial PMF fraction at 5 ppm. Three other samples were prepared similarly, except that the juice content was increased to 3, 4 and 5%, respectively, while the sugar and acid content was slightly adjusted so that the final beverage was at the same Brix and acid level. The same orange flavor was used in these samples, but no PMF fraction was added. The expert panelists were asked to compare the first sample with the other 3 samples and select which sample most closely matched the juice content of the PMF containing sample. Twenty five percent of the panelists selected the 4% sample and 50% of the panelists the 5% sample, leading to the conclusion that the PMF fraction substantially increased the apparent juice content of the 2% orange-juice containing beverage.

Conclusion

In conclusion, we have demonstrated that PMFs are naturally found in a number of different citrus products including orange and tangerine juices and peel oils. The typical levels in the juices are between 3 and 10 ppm while the peel oils contain much higher levels. Using flavor studies of the a PMF fraction in the dosage range similar to that found in nature, we can conclude that the PMFs do not have a strongly characteristic citrus flavor. However, they appear to contribute to the overall juice-like mouthfeel of orange and tangerine juices. The use of PMFs gives us an important new tool to reproduce this juice-like perception in citrus flavors.

References

1. Rusznyak, S.; Szent-Györgyi, A. *Nature* **1936**, *27*, 138.
2. Hendrickson, R.; Kesterson, J.W. *Citrus By-Products of Florida - Commercial Production Methods and Properties*; Bulletin 487, University of Florida Agricultural Experimental Station: Gainesville, FL, 1951; pp 52 – 53.
3. Kurowska, E.M.; Manthey, J.A. In *Flavonoids in Cell Function*; Buslig, B. and Manthey, J.A., Eds.; Kluwer Academic/Plenum Publishers: New York, NY, 2002; pp 173- (and reference therein).

4. Manthey, J.A.; Guthrie, N.; Grohmann, K. *Current Medicinal Chemistry* **2001**, *8*, 135-153 (and reference therein).
5. Guthrie, N.; Kurowska, E.M. PCT Patent WO 02/087567 A2, 2002.
6. Roprai, H.K.; Dexter, D.; Pilkington, G.J. PCT Patent WO 02/053152 A1, 2002.
7. Delaney, B.; Phillips, K.; Buswell, D.; Mowry, B. *et al. Food and Chemistry Toxicology* **2001**, *39*, 1087 – 1094 (and reference therein).
8. Ooghe, W.C.; Ooghe, S.J.; Detevernier, C.M.; Huyghebaert, A. *J. Ag. Food Chem.* **1994**, *42*, 2191 – 2195.
9. Manthey, J. Private communication, 1999.
10. Pupin, A.M.; Dennis, M.J.; Toledo, M.C.F. *Food Chemistry* **1998**, *63*, 513-518.
11. Gaydou, E.M.; Bianchini, J.P.; Randriamiharisoa, R.P. *J. Ag. Food Chem* **1987**, *35*, 525 – 529.
12. Manthey, J.A.; Grohmann, K. *J. Ag. Food Chem.* **2001**, *49*, 3268 – 3273.

Chapter 12

Structural and Functional Characterization of a Multimodal Taste Enhancer in Beef Bouillon

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Aimed at investigating taste enhancers in beef bouillon, taste activity-guided fractionation combined with the comparative taste dilution analysis led to the discovery of the presence of a sweet enhancing compound. Model Maillard reactions, spectroscopic and synthetic experiments revealed the previously unknown 1-(1-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt, named alapyridaine, as the first nonvolatile, tasteless sweet enhancer reported. Sensory analysis of synthetic beef taste reconstitutes spiked with synthetic alapyridaine in its “natural” concentration revealed a significant increase in sweetness, but also in the salty and umami character. Additional systematic sensory studies demonstrated for the first time that this compound is a general taste enhancer which is able to simultaneously intensify sweet, salty and umami taste modalities. Studies on the influence of the stereochemistry on sensory activity revealed the (+)-(S)-alapyridaine as the physiologically active compound, whereas the (-)-(R)-enantiomer did not show any effect.

Due to its desirable flavor, beef broth is frequently used as a savory base for processed food compositions and convenience foods. Although the consumer acceptance of such food products is strongly influenced by both the aroma-active volatiles and the taste-active non-volatile compounds, detailed information on the structures and sensory properties are as yet mainly available on the volatile odor-active compounds (1-4). In comparison to the odorants, the non-volatile components imparting the unique taste of beef broth have not been sufficiently determined. On the basis of quantitative analysis in beef broth, aqueous taste recombinates have been prepared consisting of a blend of amino acids, nucleotides, carboxylic acids, inorganic salts, creatine, creatinine, and carnosine (5,6), each in their "natural" concentrations. Sensory evaluation of these biomimetic taste imitations demonstrated that the well balanced typical taste of the authentic beef broth could not be completely reproduced only through the compounds already identified (5,6). In preliminary experiments, we compared the taste quality of an authentic beef broth with the overall taste of a broth taste recombine, and confirmed the data reported earlier (5). Besides the umami note, in particular, the intensity of the sweet taste quality of beef broth could not be sufficiently replicated by the biomimetic imitation. On the basis of these data, it has to be concluded that the attractive and unique taste of beef broth is due to yet unknown taste compounds which might be not present *per se* in beef meat, but are formed from tasteless precursors during thermal treatment.

In order to identify such thermally generated taste compounds, we recently developed the so-called Taste Dilution Analysis (TDA), which is based on the determination of the detection threshold of taste compounds in serial dilutions of chromatographic fractions (7-9), offering the possibility to rate food components according to their relative taste impact.

The objectives of the present investigation were, therefore, (i) to screen a beef broth for taste enhancing compounds by application of taste dilution analyses, (ii) to identify the key compound contributing to sweet taste, and (iii) to study its sensory impact on the taste quality of beef broth by means of taste reconstitution experiments.

Experimental

Materials

Beef bouillon was freshly prepared from beef purchased in a local shop in Germany. After cooling to 2°C, the bouillon was de-fatted by filtration, followed by extraction with ethyl acetate prior to sensory analysis and activity-

guided fractionation (10). A synthetic beef bouillon taste model system was prepared by dissolving a cocktail of 34 compounds consisting of amino acids, organic acids, sugars, minerals, nucleotides, carnosine, creatine, and creatinine in their natural concentrations in water adjusted to pH 6.0 (6, 10).

Chemicals

Racemic 1-(1-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (alapyridaine) was prepared following the procedure recently reported in the literature and was purified using food-grade solvents (11). The enantiomers (+)-(*S*)- and (-)-(*R*)-alapyridaine were stereospecifically synthesized and purified using food grade solvents (12).

Chromatographic, spectroscopic and sensory techniques

Ultrafiltration (10), gel permeation chromatography (10), comparative taste dilution analyses (10, 11), NMR and LC/MS experiments (11), and polarimetry (12) were performed following the procedures recently reported. CD spectroscopy was performed by H.-U. Humpf, Institute for Food Chemistry, University of Muenster.

Results and Discussion

In order to isolate hydrophilic, taste enhancing compounds formed during cooking of meat, beef bouillon was freshly prepared, and then de-fatted by fat crystallization, followed by solvent extraction. After removing trace amounts of solvent in vacuo, a trained sensory panel described the overall taste of the de-fatted beef bouillon as intense and complex centering around umami, saltiness, sweetness, and sourness. In order to map the key tastants and to locate potential compounds imparting to the sweetness of the beef broth, the aqueous solution was fractionated following an activity-guided procedure.

Mapping of compounds contributing to sweet taste

Aimed at removing proteins and high molecular weight, melanoidin-type material, the de-fatted beef bouillon was separated by means of ultrafiltration with a molecular weight cut-off of 1 kDa. Sensory analysis demonstrated that only the low molecular weight fraction (MW < 1kDa) exhibited the typical complex, sweet, brothy taste.

In order to analyze for sweet taste compounds in beef bouillon, the low molecular weight fraction was further separated by gel permeation

chromatography (GPC) on Sephadex G-15 using aqueous acetic acid as the eluent. Monitoring the effluent at 254 nm, the GPC chromatogram displayed in Figure 1 was recorded and ten fractions (Fractions I to X) were collected separately (10). These fractions were separately freeze-dried and the odorless residues were dissolved in the same amount of water. The aqueous solution of each fraction was then presented to the sensory panel to judge the taste qualities and intensities by application of the taste dilution analysis. To achieve this, each solution was stepwise (1+1) diluted with water and the dilutions were then presented in order of increasing concentrations to trained sensory panelists, who were asked to evaluate the taste quality and to determine the dilution at which a taste difference between the diluted fraction and two blanks (tap water) could just be detected. The panel could not detect a sweet taste in any of these fractions, thus indicating that a putative taste enhancer increasing the sweet taste of the sugars might be present in one of these fractions.

In order to analyze for putative taste modifiers enhancing the sweetness of the sugars present in beef bouillon, the fractions of another aliquot of the GPC fractionation were dissolved in the same amount of an aqueous 2-fold hyper-threshold sucrose solution. Each solution was stepwise diluted 1+1 with water until the sweetness threshold was reached. As this so-called Taste Dilution (TD) factor, obtained for each fraction, is related to its sweet taste activity in water, the ten GPC fractions were rated in their relative taste intensity (Figure 1).

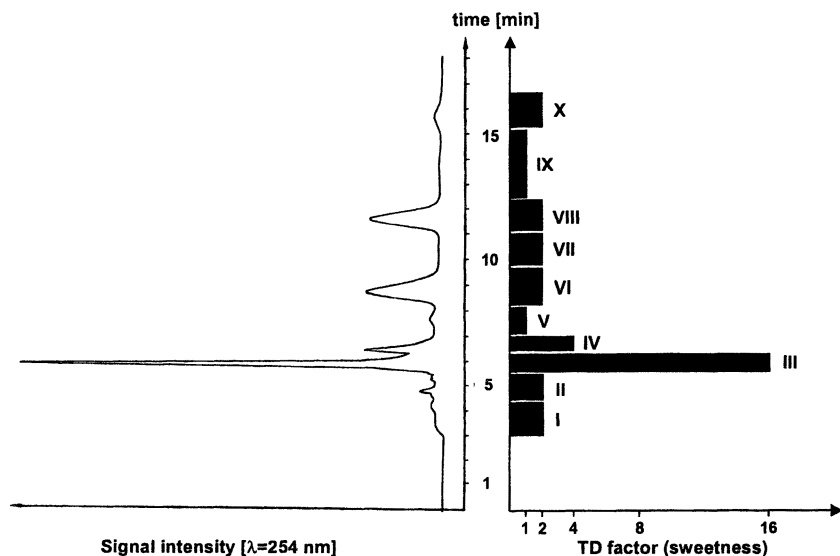


Figure 1. GPC chromatogram (left side) and taste dilution (TD) factors for sweetness (right side) of the low molecular weight fraction ($MW < 1$ kDa) isolated from de-fatted beef bouillon by means of ultrafiltration

The comparative taste dilution analysis revealed a high TD factor of 16 for sweetness in fraction III (Figure 1). As this fraction showed no sweetness in the absence of sucrose, this fraction was assumed to contain a reaction product enhancing the sweetness of the sucrose solution by a factor of eight. Because sweetness enhancing compounds were not yet reported in beef bouillon, the following identification experiments were focused on the sapid taste modifier present in GPC fraction III (Figure 1).

Identification of a sweetness enhancing compound in GPC fraction III

In order to further resolve GPC fraction III into distinct sensory active compounds and to rate them in their relative taste impacts, this fraction was then separated by RP-HPLC (Figure 2) into 16 subfractions. Application of the comparative taste dilution analysis using sucrose as the basic tastant revealed the highest TD factor for sweetness for fraction III-5, thus indicating the presence of a sweetness enhancing compound in that fraction.

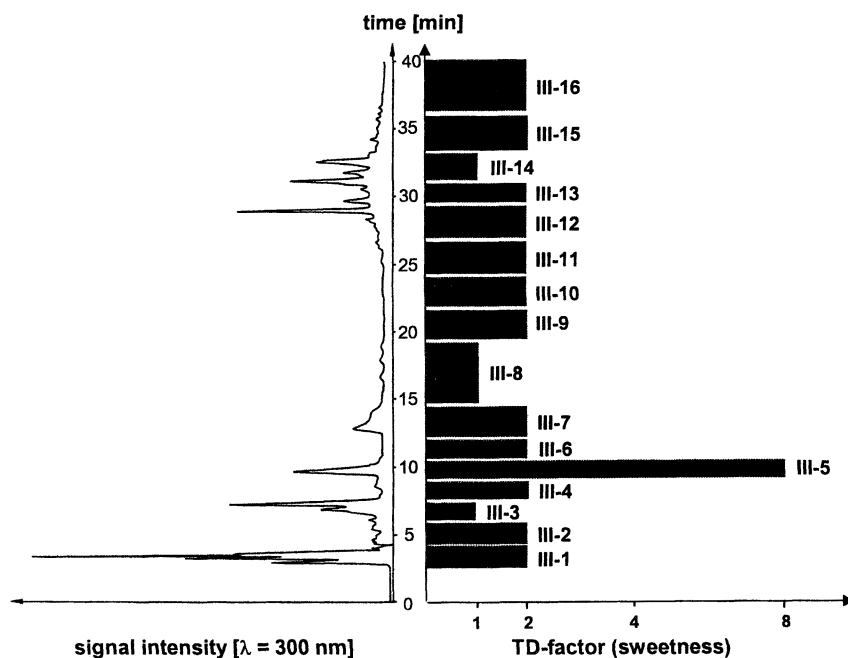


Figure 2. RP-HPLC chromatogram (left side) and Taste Dilution (TD) factors for sweetness (right side) of the GPC fraction III isolated from beef bouillon

To gain further insights into the chemical structure of the compound causing the sweet taste, fraction III-5 was separately collected and analyzed by RP-HPLC. Isolated fractions were analyzed using a diode array detector and a mass spectrometer. The compound exhibiting sweet taste activity upon degustation showed a molecular mass of 197 Da and exhibited two UV-Vis absorption maxima at 251 and 328 nm when measured at pH 8.2, or a sole maximum at 298 nm when measured at pH 3.5.

As the sweet compound present in fraction III-5 could not be detected in non-cooked beef juice (data not shown), we suggested that it might be formed upon thermal processing from non-tasting precursors in the beef meat, e.g. by Maillard reactions involving amino acids and reducing carbohydrates. Because L-alanine and hexoses are the quantitatively predominating Maillard precursors in beef juice, we compared the LC/MS and UV/Vis data, retention time (RP-HPLC), and sensory activity with those of a sweet enhancing Maillard reaction product, which was very recently isolated from a thermally treated aqueous glucose/L-alanine solution (10). On the basis of identical spectroscopic, chromatographic, and sensory data, the tastant imparting the sweetness to fraction III-5 was identified as 1-(1-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (Figure 3), and, finally, confirmed by co-chromatography of an aliquot of fraction III-5 and the synthetic reference compound (10, 11). The identification of this compound, named alapyridaine, verifies for the first time the natural occurrence of taste enhancing Maillard-type pyridinium betaines in thermally processed foods.

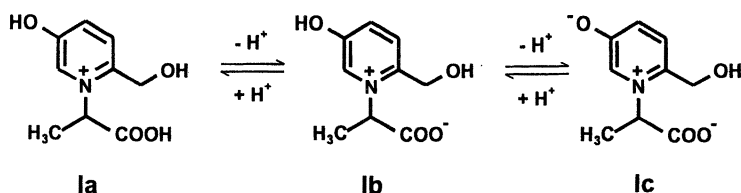


Figure 3. Protomers of 1-(1-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (alapyridaine)

Influence of the pH on structure and taste enhancing activity

To answer the question how the charge distribution in the taste enhancer is influenced by the pH value, alapyridaine was analyzed by UV/Vis as well as ^{13}C NMR spectroscopy in aqueous solution at pH 2.0, 5.0, and 7.0. The results of the UV/Vis measurements revealed that the absorption spectrum of the Maillard compound in aqueous solution is strongly influenced by the pH value, e.g. the

UV/Vis spectrum at pH 7.0 exhibited two absorption maxima, one at $\lambda = 251$ nm, the other at 328 nm (left in Figure 4). Lowering the pH to 5.0 revealed a decrease in the absorption at $\lambda = 251$ and 328 nm, and led to the detection of an additional maximum at $\lambda = 298$ nm (left in Figure 4). In contrast, only a sole maximum at $\lambda = 298$ nm was detectable at pH 2.0 (left in Figure 4).

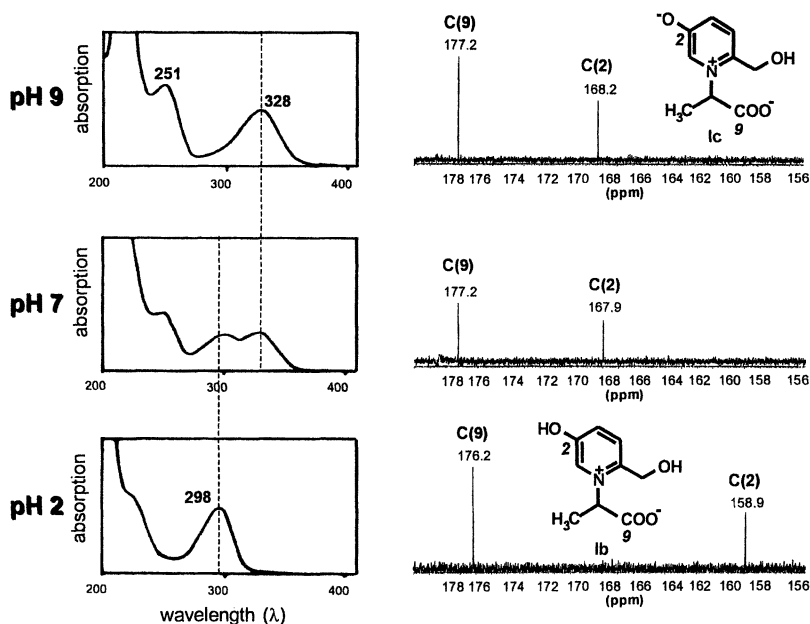


Figure 4. Influence of the pH value on the UV/Vis spectrum (left side), and the ^{13}C NMR data (excerpt on right side) of 1-(1-carboxyethyl)-5-hydroxy-2-(hydroxymethyl)-pyridinium inner salt (alapyridaine)

Measuring the ^{13}C NMR spectrum of an aqueous solution of the pyridinium betaine at pH 7.0 showed a resonance signal at 177.2 and 167.9 ppm corresponding to the carboxy group C(9) and the quarternary oxygen-bound carbon C(2) in the pyridinium moiety (right in Figure 4). Decreasing the pH value to 5.0 induced a high-field shift of the resonance signal of C(2) by 1.8 ppm, whereas the chemical shift of C(9) was only slightly affected (right in Figure 4), thus indicating that the oxygen atom in the pyridinium-3-olate moiety starts to be protonated by lowering the pH. At pH 2.0, the chemical shift of the resonance signal of C(2) was shifted by 7.2 ppm from 166.1 to 158.9 ppm, whereas the signal of the carboxy group C(9) was high-field shifted only by 0.8 ppm (right in Figure 4). Taking all these data into account, it can be summarized

that, strongly depending on the pH value, alapyridaine exists in an equilibrium of the three structures 1a - 1c displayed in Figure 3. The data also indicate that in both neutral and alkaline media, the carboxy group in the alanine moiety as well the oxygen atom of the pyridinium-3-olate moiety, are de-protonated (11). By lowering the pH to 5.0, first the oxygen group in the pyridinium-3-olate moiety is protonated, being reflected by the hypsochromic shift of the absorption maximum from $\lambda = 328$ to 298 nm, and the high-field shift of the resonance signal of C(2) in the ^{13}C NMR spectrum. Thereafter, the carboxy function starts to be protonated at very low pH values which are, however, not relevant for food products (data not shown).

In order to study the influence of the pH value on the sweetness enhancing effect of the novel Maillard reaction product, binary mixtures of the tasteless alapyridaine and the sweet tasting compounds glucose, saccharose, or L-alanine were sensorially evaluated in a triangle test using water with pH 5.0, 7.0 and 9.0, and the sweet detection thresholds determined were compared to the threshold concentrations of aqueous solutions containing the sweet tastants alone (Table I).

Table I. Effect of Alapyridaine on the Detection Thresholds of Sweet Tasting Compounds

compounds	pH	detection threshold ^a [mmol/kg water]	threshold decrease ^b
glucose	5/7	48.0	
+ alapyridaine (1/1)	5	6.0	8
+ alapyridaine (1/1)	7	3.0	16
+ alapyridaine (1/1)	9	3.0	16
saccharose	5/7	12.5	
+ alapyridaine (1/1)	5	6.0	2
+ alapyridaine (1/1)	7	3.0	4
+ alapyridaine (1/1)	9	1.5	8
L-alanine	7	12.0	
+ alapyridaine (1/1)	5	6.0	2
+ alapyridaine (1/1)	7	3.0	4
+ alapyridaine (1/1)	9	1.5	8

^a The taste threshold concentrations were determined by a triangle test using tap water (pH 5.0, 7.0, 9.0) as the solvent. ^b Factor by which the threshold of the sweet tastant was decreased in the presence of alapyridaine.

Depending on the pH value, the detection threshold of glucose was significantly decreased when alapyridaine was present, e.g. the threshold dropped from 48.0 mmol/L by a factor of 16 to 3.0 (11, 13). Lowering the pH value to 5.0 led to a less pronounced effect, e.g. only a threshold decrease by a factor of 8 was detectable. Furthermore, the sensory panel was able to detect the sweet taste of the disaccharide saccharose as well as of the amino acid L-alanine in four- and eight-fold lower concentrations at pH 7.0 and 9.0, respectively, when alapyridaine was present (Table I). Correlating well with the data found for glucose, the sweet enhancing effect was more pronounced at pH 7.0 and 9.0 than at pH 5.0. Since at pH>5, the alapyridaine was shown by UV/vis and ¹³C NMR to be present as the anion 1C (Figure 3), this strong pH dependency of the sensory activity of the alapyridaine indicates that the deprotonated pyridinium-3-olat, 1C, can be considered as the physiologically active form of alapyridaine.

Influence of alapyridaine on the taste of beef bouillon

In order to investigate the contribution of alapyridaine to the overall taste of a beef broth, first, the natural amount of the tastant was determined quantitatively in an authentic beef broth. Quantitative HPLC analysis was, therefore, performed on GPC fraction III by comparing the peak area obtained at $\lambda = 298$ nm with those of a defined standard solution of the reference compound in water. Calculating the mean from triplicate tests, the concentration of alapyridaine in the beef broth was found to be 419 $\mu\text{g/L}$ (10).

In order to prove the results of the chemical analysis and to check whether the Maillard product contributes to the typical taste of beef broth taste, we investigated whether the "natural" amount of alapyridaine in beef broth is sufficient to impart a sensory effect to a synthetic blend of taste chemicals present in their authentic concentrations. To achieve this, we, first, prepared an aqueous taste recombinant consisting of 16 amino acids, four sugars, three 5'-nucleotides, three organic acids, three salts, phosphate, carnosine, creatine and creatinine, each in their natural concentrations present in beef broth, and asked a trained sensory panel to score the taste descriptors given in Figure 5 on a scale from 0 (not detectable) to 5 (strongly detectable). Sensory evaluation of this taste recombinant revealed the highest intensity for the sour note (4.0), closely followed by a salty (3.0) and umami-like taste quality (3.0) (Figure 5A). In contrast, sweetness was judged to only have an intensity of 1.0. The panelists concluded that the typical taste of an authentic beef broth, which showed increased intensity in the umami (4.2) and sweet character (1.6), and less intensity in sourness (2.4), cannot be completely reconstituted only by the blend of compounds already identified.

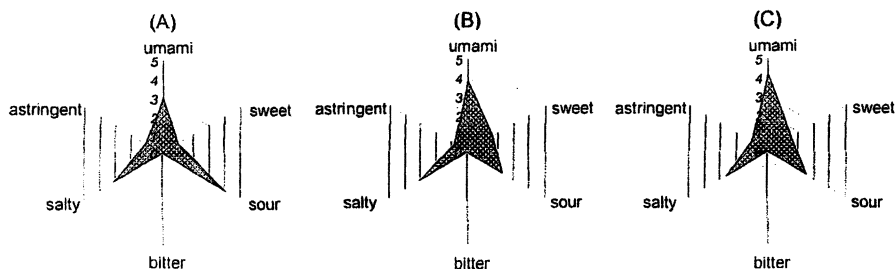


Figure 5. Taste profiles of (A) beef taste recombine, (B) beef taste recombine plus “natural” concentration of alapyridaine (419 $\mu\text{g/L}$), and (C) authentic beef bouillon

In order to check the influence of alapyridaine on the overall taste of the beef recombine, synthetic alapyridaine was added in its “natural” concentration of 419 $\mu\text{g/L}$ to the taste recombine. The overall taste of that solution was compared with that of the taste recombine lacking in the alapyridaine. As given in Figure 5B, the sensory panel perceived an increase in sweetness (1.7) and umami character (3.8) and, in parallel, a slight decrease in the sour note in the recombine, thus demonstrating that, in authentic concentrations, the alapyridaine exhibited a pronounced effect on the overall taste quality of beef broth (10). These data demonstrate for the first time, that the alapyridaine is active in modulating both, sweet and umami taste of real beef broth when present in “natural” concentrations. Comparing the taste profiles of the blend of taste chemicals including the alapyridaine (Figure 5B) with that of an authentic beef broth (Figure 5C) demonstrated furthermore that the individual taste qualities could be mimicked quite well in their intensities. This demonstrates that the naturally occurring alapyridaine is an important key contributor to the desirable taste of beef broth.

Influence of the stereochemistry on taste enhancing activity

In spite of using enantiopure alanine as the starting material, **1** was obtained as a racemate under reflux conditions in alkaline aqueous solutions. To study the racemization process, L-alanine or racemic **1** was refluxed in D_2O at pD 7.0 for 24 h. No proton-deuterium exchange was observed for L-alanine by ^1H NMR (data not shown). However, the data obtained for racemic **1** indicated the disappearance of the enantiotopic α -proton (Figure 6). Compared to L-alanine, the acidity of the proton at the chiral center is increased by the pyridinium moiety. Abstraction of the α -proton results in the formation of a carbanion at the

asymmetric carbon, thus facilitating the racemization (12). Additional experiments focusing on the stereochemistry of the alanine moiety in alapyridaine by means of polarimetry showed that the Maillard product is formed as a 1:1 racemic mixture of the (*R*)- and the (*S*)-enantiomer of 1.

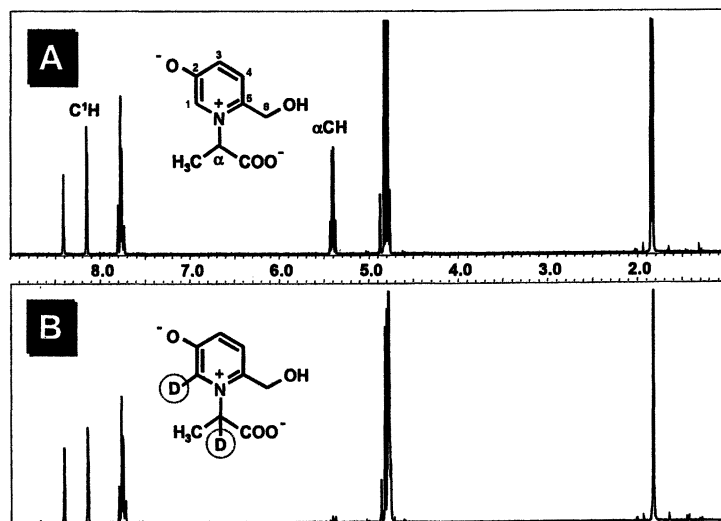


Figure 6. ^1H NMR spectrum (400 MHz) of alapyridaine prior (A), and (B) after heating for 12h in D_2O (pD 7.0)

Aimed at investigating the taste enhancing activity of the individual enantiomers of alapyridaine, enantiopure 1 was prepared upon reductive amination of 5-(hydroxymethyl)-2-furaldehyde and L-alanine with Raney nickel/hydrogen. This reaction resulted in the corresponding (*S*)-*N*-(1-carboxyethyl)-2-hydroxymethyl-5-(methylamino)furan (Figure 7). The latter was converted into the target pyridinium betain compound by mild oxidation with bromine in water/methanol to yield (+)-(*S*)-1. Similarly, the reaction with D-alanine resulted in (-)-(*R*)-1. After purification, the presence of (*S*)-1 and (*R*)-1 was proven by polarimetry, revealing optical rotations of $+40.2^\circ$ and -38.6° , respectively (12).

A final proof for the stereochemistry present in alapyridaine was performed by CD spectroscopy. As shown in Figure 8, the alapyridaine sample isolated from heated Maillard mixtures did not show any CD effect, thus confirming the formation of racemic alapyridaine during thermal treatment as suggested by the optical rotation of 0° measured by polarimetry. In contrast, the synthetic (*S*)-(+)-enantiomer showed a pronounced CD effect, in particular, when measured at pH 9, thus confirming the enantiopurity of that sample.

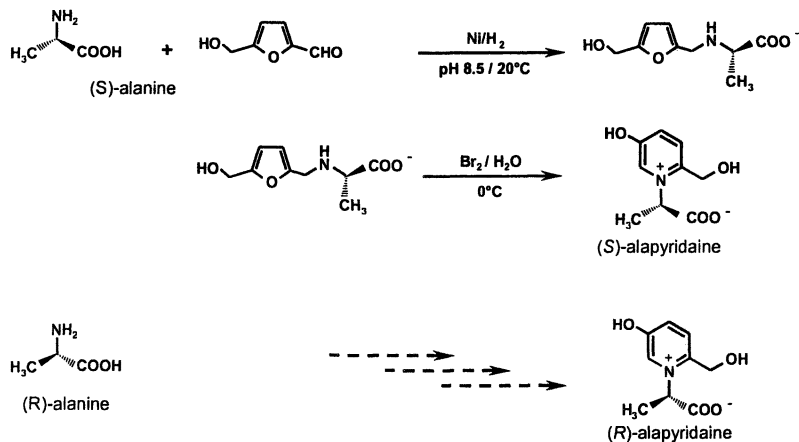


Figure 7. Synthetic preparation of (+)-(S)- and (-)-(R)-alapyridaine

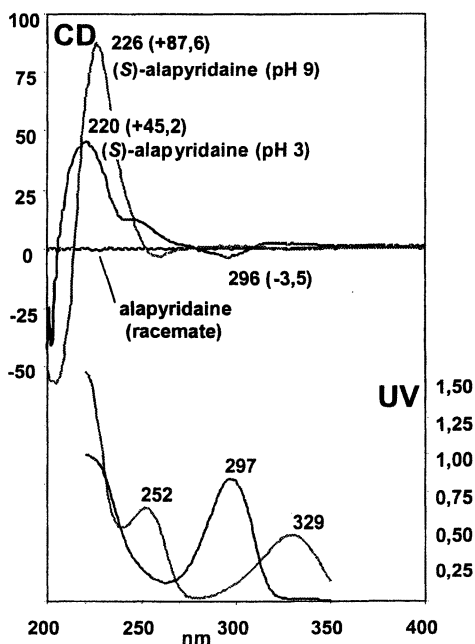
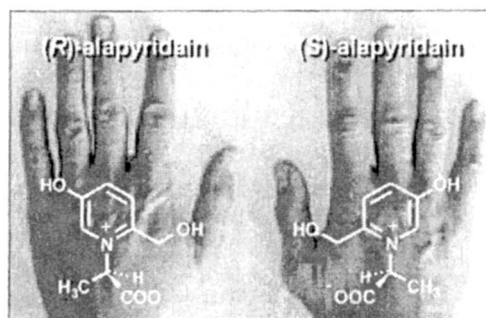


Figure 8. UV-Vis and CD spectroscopy of racemic and (+)-(S)-alapyridaine. (S)-alapyridaine at pH 3 has a UV maximum at 297nm and (S)-alapyridaine at pH 9 has UV maxima at 252 and 329 nm.

In order to investigate the influence of the stereochemistry of alapyridaine on sweetness perception, we investigated the influence of (+)-(*S*)- and (-)-(*R*)-alapyridaine on the threshold concentration of the monosaccharide glucose at pH 7.0 (Figure 9). The (+)-(*S*)-alapyridaine was found to be twice as efficient as the racemic mixture (c.f. Table I). For example, the threshold of glucose was decreased by a factor of 32. In contrast, the (-)-(*R*)-enantiomer did not affect the sweetness threshold of the sugar (13).



alapyridaine-induced factor of threshold decrease for:

glucose	(sweet)	<1	× 32
monosodium glutamate	(umami)	<1	× 8
sodium chloride	(salty)	<1	× 5
caffeine	(bitter)	<1	<1
citric acid	(sour)	<1	<1

Figure 9. Influence of the stereochemistry of alapyridaine on the recognition threshold of basic taste compounds

Determination of the recognition thresholds of aqueous solutions of the umami tasting monosodium glutamate (MSG) and the salty tasting sodium chloride (NaCl), respectively, in the absence or presence of equimolar amounts of the alapyridaine enantiomers revealed that the detection thresholds of MSG (1.5 mmol/L) and NaCl (10 mmol/L) were 8 and 5 times lower when (+)-(*S*)-alapyridaine was present (13). On the basis of these data it might be concluded that alapyridaine is a multivalent taste enhancer increasing the human sensitivity for sweetness, saltiness, and umami taste.

Comparative sensory studies on the role of alapyridaine in modifying bitter and sour taste perception revealed that neither the threshold concentrations of the bitter compounds L-phenylalanine and caffeine, nor that of the sour tasting citric acid were influenced by the presence of alapyridaine (13).

Relative taste enhancing factor in iso-intense solutions

Finally, iso-intensity experiments were performed to determine how much the concentration of sucrose, MSG and NaCl can be reduced in the presence of alapyridaine to meet the same taste intensity as aqueous solutions containing the basic tastants alone. The iso-intensity experiments revealed that the relative sweetness of the mixtures containing sucrose and alapyridaine increases with increasing amounts of the novel taste modifier (Figure 10). This shows again the enhancing potential of alapyridaine on the sweet taste of sucrose; for example, a solution containing 20 mmol/L sucrose and 20 mmol/L alapyridaine has to be diluted 4-fold to match the sweetness of a solution containing 20 mmol/L sucrose only (13). Further increase of the alapyridaine concentration, however, revealed a slight reduction in taste enhancing efficiency. For example, in the presence of 30 mmol/L alapyridaine a relative sweetness factor of 3 was determined.

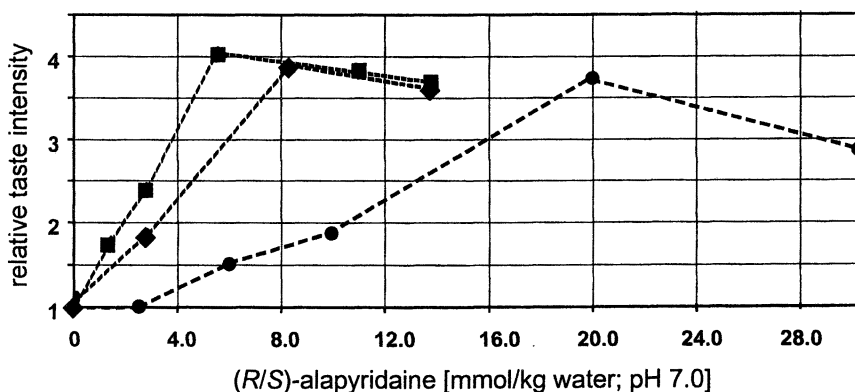


Fig. 10. Relative taste enhancing factor (Y axis) of (R/S)-alapyridaine in iso-intensely tasting solutions (pH 7.0) of (●) sucrose (20 mmol/L) and mixtures containing sucrose (20 mmol/L) and (R/S)-alapyridaine in various concentrations, (■) MSG (3.0 mmol/L) and mixtures containing MSG (3.0 mmol/L) and (R/S)-alapyridaine in various concentrations, (◆) NaCl (20 mmol/L) and mixtures containing NaCl (20 mmol/L) and (R/S)-alapyridaine in various concentrations.

To gain information about how much the NaCl concentration can be reduced in the presence of alapyridaine, iso-solution experiments were performed with aqueous solutions of NaCl and alapyridaine. As outlined in Figure 10,

increasing the alapyridaine concentration increased the saltiness perception of the 20 mmol/L NaCl solution. The increase in saltiness perception ran through a maximum at a concentration of 10 mmol/L alapyridaine, thereafter slightly decreasing again (13).

A corresponding experiment with an aqueous solution of MSG (3.0 mmol/L) revealed that the umami intensity of the mixtures containing MSG and alapyridaine increases with rising amounts of alapyridaine up to a concentration of 6 mmol/L (Figure 10). A solution containing 3.0 mmol/L MSG and 6.0 mmol/L alapyridaine has to be diluted by a factor of 4 to match the umami intensity of a solution containing only 3.0 mmol MSG (Figure 10). This means that a solution containing 0.75 mmol/kg MSG and 1.5 mmol/kg alapyridaine has the same umami intensity as the umami standard solution (3.0 mmol/L).

Influence of alapyridaine on perception of tastant mixtures

The following experiments were aimed at investigating the effect of alapyridaine on the taste perception of mixtures of taste compounds. In a final experiment, the influence of alapyridaine on a quaternary tastant mixture containing sucrose (12.5 mmol/L), MSG (1.5 mmol/L), NaCl (10 mmol/L), and caffeine (2.0 mmol/L) was investigated (Figure 11).

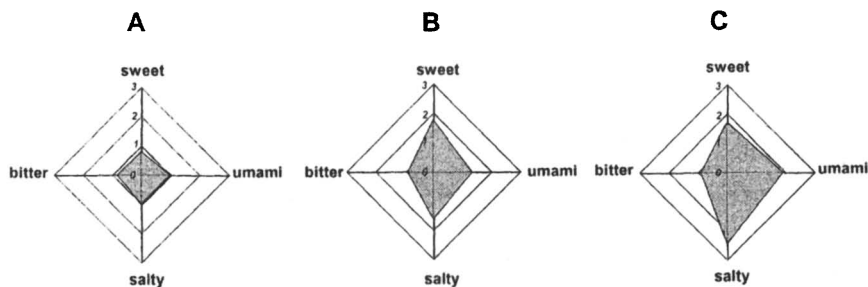


Figure 11. Influence of (R/S)-alapyridaine on the taste intensities of a tastant mixture. Aqueous solutions (pH 7.0) of sucrose (12.5 mmol/L), MSG (1.5 mmol/L), NaCl (10 mmol/L), and caffeine (2.0 mmol/L) were presented in the absence (A) and presence of 1.5 mmol/L (B), or 7.5 mmol/L (C) of (R/S)-alapyridaine.

The taste intensities for the individual taste qualities were centered around a score of 1.0 when alapyridaine was absent. The sensorial evaluation of this quaternary solution in the presence of 1.5 mmol/L alapyridaine revealed a significant increase, in particular, of the intensity of sweetness and umami taste, and to a somewhat lower extent of saltiness, but the bitter taste was not affected

(13). Addition of 7.5 mmol/L alapyridaine showed a more pronounced effect for the salty taste, which was rated with a score of 2.5. Also, the umami taste was further intensified to some extent, but no further enhancement of sweetness was detectable. Again, the bitter taste of caffeine was not affected.

Taking all these data into consideration, it can be concluded that (+)-(S)-alapyridaine present in beef bouillon does enhance not only the intensity of a single taste modality, but increases the human sensitivity for sweetness, saltiness and umami taste, thus illustrating alapyridaine as a naturally occurring, multivalent taste enhancer.

References

1. Gasser, U.; Grosch, W. *Z. Lebensm. Unters. Forsch.* **1990**, *190*, 511-515.
2. Guth, H.; Grosch, W. *J. Agric Food Chem.* **1994**, *42*, 2862-2866.
3. Cerny, M.; Grosch, W. *Z. Lebensm. Unters. Forsch.* **1992**, *194*, 322-325.
4. Cerny, M.; Grosch, W. *Z. Lebensm. Unters. Forsch.* **1993**, *196*, 417-422.
5. Warendorf, T.; Belitz, H.D.; Gasser, U.; Grosch, W. *Z. Lebensm. Unters. Forsch.* **1992**, *195*, 215-223.
6. Shima, K.; Yamada, N.; Suzuki, E.-I.; Harada, T. *J. Agric. Food Chem.* **1998**, *46*, 1465-1468.
7. Frank, O.; Ottinger, H.; Hofmann, T. *J. Agric. Food Chem.* **2001**, *49*, 231-238.
8. Frank, O.; Hofmann, T. *J. Agric. Food Chem.* **2002**, *50*, 6027-6036.
9. Ottinger, H.; Bareth, A.; Hofmann, T. *J. Agric. Food Chem.* **2001**, *49*, 1336-1344.
10. Ottinger H., Hofmann T. *J. Agric. Food Chem.* **2003**, *51*, 6791-6796.
11. Ottinger, H.; Soldo, T.; Hofmann, T. *J. Agric. Food Chem.* **2003**, *51*, 1035-1041.
12. Villard R., Robert F., Blank I., Bernardinelli G., Soldo T., Hofmann T. *J. Agric. Food Chem.* **2003**, *51*, 4040-4045.
13. Soldo T., Blank I., Hofmann T. *Chem. Senses* **2003**, *28*, 371-379.

Chapter 13

Chocarom Pyrazine: A Remarkable Pyrazine for Flavors and Fragrances

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Chocarom Pyrazine is a colorless transparent liquid consisting of an isomeric mixture of 3,5-dimethyl-2-isobutylpyrazine and 3,6-dimethyl-2-isobutylpyrazine. Chocarom Pyrazine isomers were isolated from several natural sources, *e.g.* skin and flesh of potato cultivars after baking. The odor threshold of 3,5-dimethyl-2-isobutylpyrazine and 3,6-dimethyl-2-isobutylpyrazine indicates a relatively low ϕ value. The odor and flavor of Chocarom Pyrazine is reminiscent of the warmth of cocoa and hazelnut. It is slightly musky, animalic, with patchouli and vetiver tones and a mentholic note. The main uses of Chocarom Pyrazine for food are for chocolate, cocoa, baked goods, breakfast cereals, milk products, roasted and processed vegetables, soups, baked potatoes, sweet sauces and mint flavors. This unique pyrazine can be used in fragrances. It has a very unique value in fragrance compositions. It imparts a strong and natural, original and long lasting effect. A beautiful tool for women's as well as men's fragrance creations, it boosts woody notes, chypre, oriental and fougere. It is also very useful in floral accords for providing depth and warmth.

Pyrazines are materials obtained from Maillard reactions as by-products of the browning reaction of sugars and proteins or amino acids. These reactions occur during roasting, cooking, baking, etc. of different food products. Pyrazines are among the most widely distributed heterocyclic compounds in nature, and often combined with other functional groups e.g. alkyl, acetyl, alkoxy and thioalkoxy. Pyrazines containing these chemical functionalities are present mainly in roasted products, generally thermally treated, including cocoa, coffee, barley, popcorn, peanuts, other nuts, bread, potato and beef. Over seventy pyrazines containing only alkyl groups have been identified in heated foods (1). Alkylpyrazines, which are important flavor contributors, among other flavor compounds in chocolate flavor, are formed via the Maillard reaction during the roasting process of cocoa production (2). Many of the pyrazines have a nutty aroma similar to that of peanuts (3).

The importance of these materials motivated organic chemists to synthesize and use them in the flavor industry as ingredients in flavor formulations for roasted nuts, chocolate, meat flavors, etc. By 1970, the first pyrazines obtained GRAS status in the US, for use as flavoring substances, and today many pyrazines are commercially available for food applications.

Results and Discussion

Chocarom Pyrazine is a colorless transparent liquid consisting of a 1:1 isomeric mixture of 3,5-dimethyl-2-isobutylpyrazine and 3,6-dimethyl-2-isobutylpyrazine, as shown in Figure 1. Relevant CAS numbers are [38888-81-2] for dimethyl-isobutylpyrazine (positions not defined), and [70303-42-3] for 3,5-dimethyl-2-isobutylpyrazine.

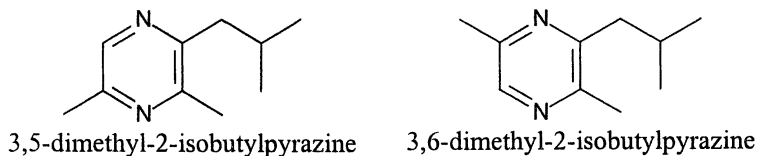


Figure 1. Chocarom Pyrazine isomers

Chocarom Pyrazine isomers were isolated from the skin and flesh of potato (*Solanum tuberosum* L.) cultivars after baking (4). 3,5-Dimethyl-2-isobutylpyrazine [2,5-dimethyl-3-(2-methylpropyl)pyrazine] was isolated by Oruna-Concha, Craig, Duckham and Ames from the following potato cultivars – *Cara*, *Nadine*, *Flanna* and *Marfona*. 3,6-Dimethyl-2-isobutyl-pyrazine [3,5-dimethyl-2-(2-methylpropyl)pyrazine], was found by the same team in *Cara* and *Marfona* potato cultivars. 2,5-Dimethyl-3-isobutylpyrazine was also detected by Welty, Marshall and Grun in chocolate ice cream prepared from cocoa flavor (5). Both pyrazines were also found as key odorant compounds in dark chocolate by Counet, Callemien, Ouwerx and Collin (6). The role of amino acids in alkyl-substituted pyrazines formation in model systems containing pyruvaldehyde was examined by Mea (7). 2,5-Dimethyl-3-isobutylpyrazine was formed in the model system with valine. Both isomers were prepared synthetically by Chen (8) by reacting acetol, isobutyraldehyde and ammonium acetate, with low yield of 22.3%. Subsequent proprietary work by the author has improved the yield to 65%.

The odor threshold of 3,5-dimethyl-2-isobutylpyrazine and 3,6-dimethyl-2-isobutyl-pyrazine was published by Wagner, Czerny, Bielohradski and Grosh, to be >2000 ng/l air (9), which indicates a relatively low ϕ value (11), of only *ca.* 82,000.

The odor and flavor of Chocarom Pyrazine is reminiscent of the warmth of cocoa and hazelnut. It is slightly musky animalic, with patchouli and vetiver tones and a mentholic note. The odor character is displayed graphically in Figure 2.

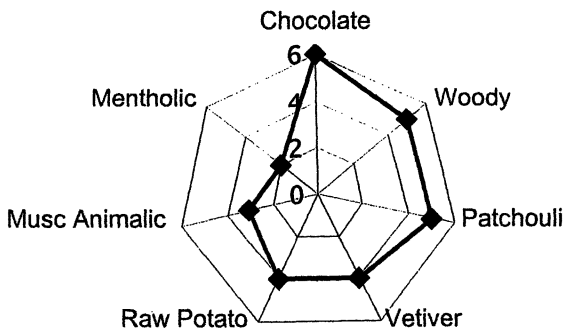


Figure 2. Radar organoleptic description of Chocarom Pyrazine

The main uses of Chocaron Pyrazine for food are for chocolate, cocoa, baked goods, breakfast cereals, milk products, roasted and processed vegetables, soups, baked potatoes, sweet sauces and mint flavors.

Table I. Acceptable Daily Intake for Chocaron Pyrazine (10)

Food Category	Mean Consumption g/day*	Anticipated Usual Use Level (ppm)	Anticipated Maximum Use Level (ppm)	PADI mg/person/d **
Baked Goods	137.2	1.5	2.0	0.21
Breakfast Cereals	20.0	1.5	2.0	0.03
Milk Products	39.5	2.0	3.0	0.08
Processed Vegetables	85.0	0.5	1.5	0.04
Seasonings & Flavors	0.01	5000	10000	0.05
Soups	31.7	0.5	1.5	0.02
Sweet Sauce	6.8	2.0	3.0	0.01

* Based on MRCA mean frequency of eating and USDA mean portion size

**PADI, mg/person/day calculated by: (Mean consumption, grams/day) x (Anticipated Usual Use Level, ppm) x 0.001

REFERENCE: Market Research Corporation of America (MRCA), in conjunction with the "Food intake and nutritive value of the diets of men, women, and children in the United States, Spring 1965", a preliminary report by the consumer and Food Economics Research Division, Agricultural Research Service, United States Department of Agriculture

Chocaron Pyrazine is a rich chocolate building-block for bakery, dairy products confectionery and ice-cream flavors. The recommended dosage is 500-3000 ppm in the final product, as described in Table I.

Chocaron Pyrazine has a unique value in fragrance compositions; It imparts a strong and natural, original and long lasting effect. Chocaron Pyrazine is a beautiful tool for women's as well as men's fragrance creations. It boosts woody notes, chypre, oriental and fougere. It is also very useful in floral accords for providing depth and warmth.

One of the most impressive perfumery creations using Chocaron Pyrazine is Tonkinarom, a fragrance base which has the following

olfactive profile: animalic, macrocyclic, and polycyclic-free musk. It is very unique due to the utilization of Chocarom Pyrazine that provides a typical effect of the musk absolute from the pouches of the Musk Deer. Tonkinarom was formulated in 2 variations, the second being free either of animalic derived components or polycyclic musks.

ϕ Value of Chocarom Pyrazine

The ϕ value of flavor and fragrance ingredients, provides a better appreciation of the odor intensity of a single molecule, taking into consideration its molecular mass (MM). It is analogous to ϵ value in UV/vis data of individual molecules. The ϕ value of a single molecule is defined as following:

$$\phi = \frac{\text{MM} \times 10^3}{\text{Threshold (ppm)}}$$

The subsequent table (Table II) lists the ϕ values for some commercially important pyrazines.

Table II. ϕ Values for Some Commercially Important Pyrazines

SUBSTANCE	MOLECULAR	
	MASS	ϕ VALUE
2-Methylpyrazine	94.11	941
2,3-Dimethylpyrazine	108.14	3,090
2,5-Dimethylpyrazine	108.14	60,078
2,6-Dimethylpyrazine	108.14	72,093
2-Ethylpyrazine	108.14	18,023
2-Ethyl-5-methylpyrazine	122.16	732,989
2-Ethyl-6-methylpyrazine	122.16	1,221,600
2,3,5-Trimethylpyrazine	122.16	305,400
2-Methoxy-3-methylpyrazine	124.14	24,828,000
2-Methoxy-3-isobutylpyrazine	166.22	831,100,000
2-Acetylpyrazine	122.13	1,969,839
3,6-Dimethyl-2-isobutylpyrazine	164.25	82,125
3,5-Dimethyl-2-isobutylpyrazine	164.25	82,125

As seen in the table, both Chocarom Pyrazine isomers, *i.e.* 3,6-dimethyl-2-isobutylpyrazine and 3,5-dimethyl-2-isobutylpyrazine, have ϕ values of *ca.* 82,000, thus belonging to the low ϕ value pyrazines group. This characteristic shows more clearly the pyrazines odor impact, as shown in the following graphs of low, medium and

high ϕ value pyrazines. Low odor impact pyrazines are defined as having ϕ values up to 250,000, e.g. 2,5-dimethylpyrazine; medium odor impact pyrazines have ϕ values of 250,000 to 5,000,000, e.g. 2-acetylpyrazine and high impact odor pyrazines, e.g. 2-methoxy-3-isobutylpyrazine, having ϕ value of 24,828,000. This concept is shown also by Figures 3 - 6.

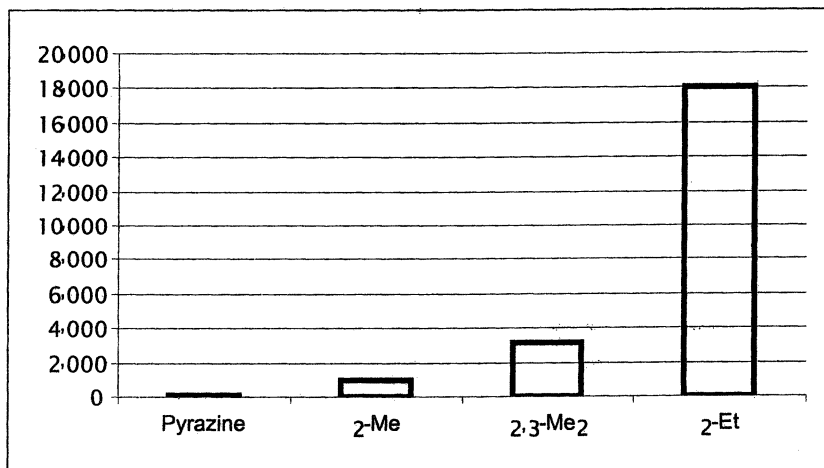


Figure 3. Low ϕ value pyrazines

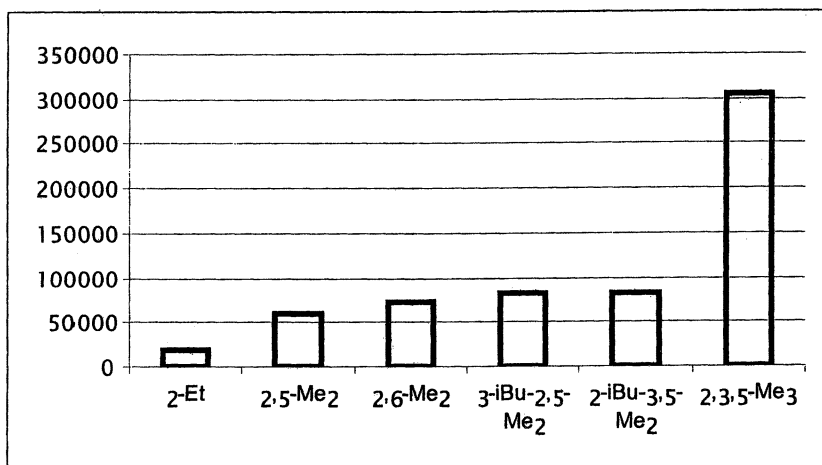


Figure 4. Medium-Low ϕ value pyrazines

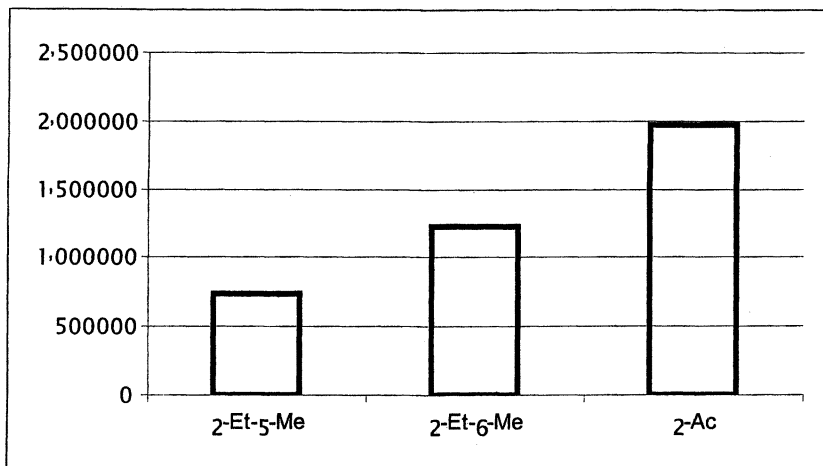


Figure 5. Medium-High ϕ value pyrazines

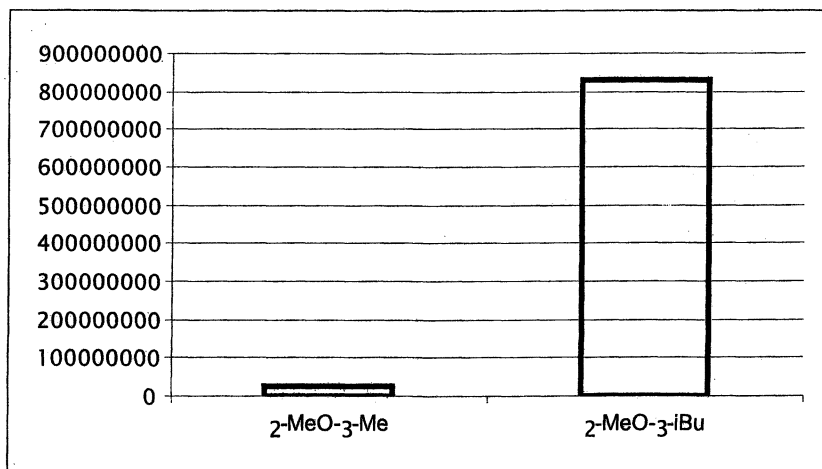


Figure 6. High ϕ value pyrazines

Physical Data of Chocarom Pyrazine

Chocarom Pyrazine is a colorless transparent liquid, insoluble in water. In ethyl alcohol 50% it is soluble from 1:1 to 1:10.

Boiling Point: 73-75°C at 2 mmHg

Flash Point: 75°C (cc)

Specific Gravity $D^{20/20}$: 0.924 – 0.934

Refractive Index n_D^{20} : 1.489 – 1.494

Stability Data of Chocarom Pyrazine

Chocarom Pyrazine was kept at 15°C for 55 days in order to measure changes in its physical parameters and odor profile. The color changed to 70 APHA (40 APHA start point); no change was seen in other physical parameters and odor profile. In another experiment Chocarom Pyrazine was kept at 40°C for 22 days. The color changed to 70 APHA (40 APHA start point); no change in other physical parameters and odor profile.

Analyses of Chocarom Pyrazine

The structure and composition of Chocarome pyrazine was elucidated by a combination of GC, MS and NMR analyses (Figures 7 and 8 and tables III - VI). The substitution pattern of 3,5-dimethyl-2-isobutylpyrazine and 3,6-dimethyl-2-isobutyl-pyrazine was elucidated by a combination of NMR methods, especially in mixtures by gradient selected ^1H , ^{15}N HMBC experiments at natural abundance level (11).

Since the ^{15}N -NMR spectrum of pyrazines was proven to be a powerful tool for their structural elucidation (12, 13) it is used to study these type of molecules. Tables V and VI details the ^{15}N -NMR chemical shift of both nitrogen atoms of each isomer:

Conclusion

Chocarom Pyrazine is a remarkable pyrazine for flavors and fragrances. The diverse organoleptic characteristics of this outstanding pyrazine, having the warmth of cocoa and hazelnut. It is slightly musky-animalic in character, with patchouli and vetiver tones and a mentholic note, enabling its use as an important building block in perfumer and flavorist imaginations for preparing innovative formulations.

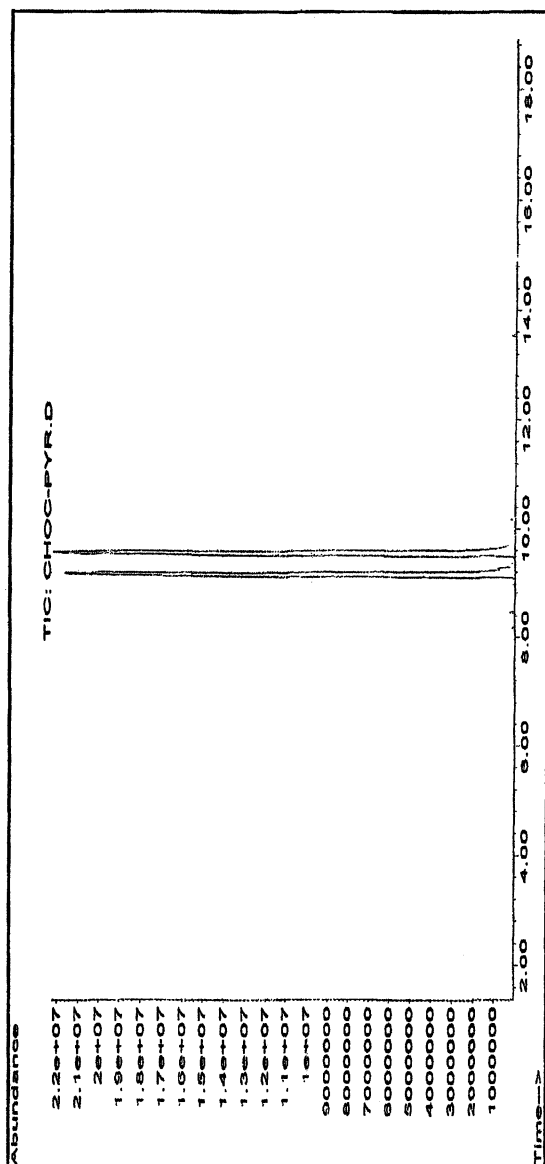


Figure 7. GC of Chocarom Pyrazine

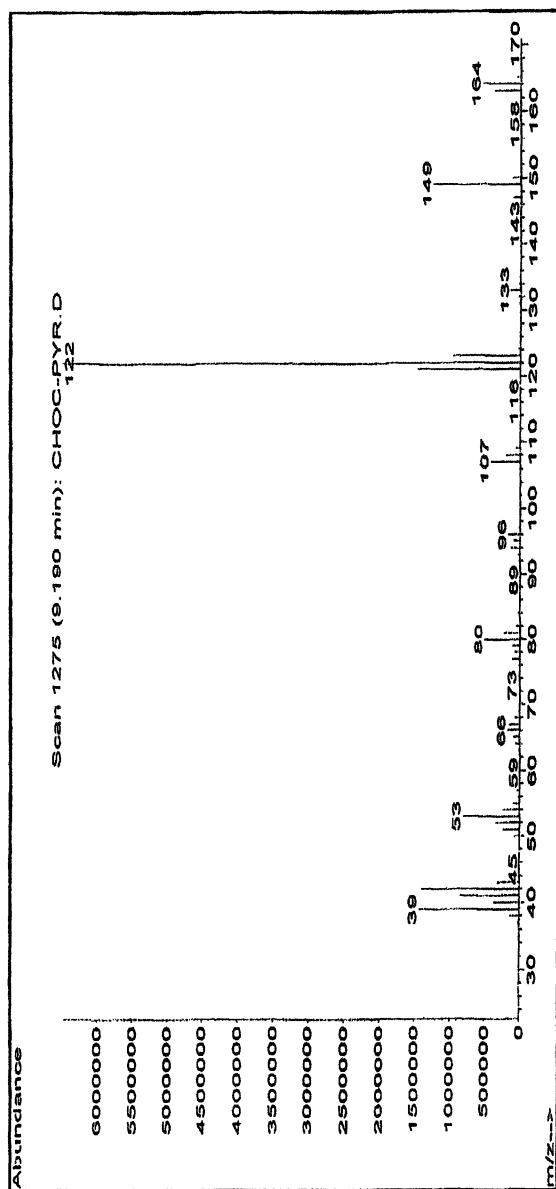


Figure 8. MS of Dimethylisobutylpyrazine

Table III. MS Fragmentation of Chocarom Pyrazine

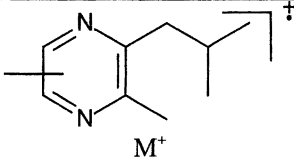
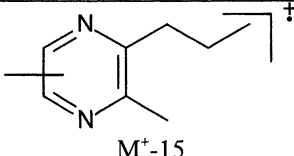
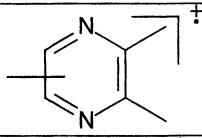
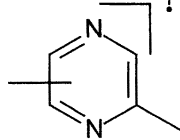
m/z	Species	Abundance
164.05	 M ⁺	522432
149.05	 M ⁺ -15	976832
122.05		6334976
107.05		245312
57.00	C ₄ H ₉ ⁺	52808
43.00	C ₃ H ₇ ⁺	250880

Table IV. ^1H and ^{13}C NMR Data for Both Isomers

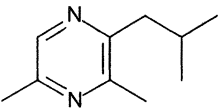
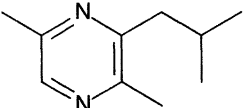
PYRAZINE	^1H Chemical Shift δ [ppm], multiplicity, Coupling Constant(s) [Hz], integral, position,	^{13}C Chemical Shift δ [ppm], position
 3,5-Dimethyl-2-isobutyl-	8.211, brs, 1 H, H6; 2.660, d, 7.3, 2 H, CH ₂ ; 2.532, d, 0.4, 3 H, C3-CH ₃ ; 2.488, s, 3 H, C5-CH ₃ ; 2.130, mc, 1 H; CH; 0.954, d, 6.6, 6 H, CH(CH ₃) ₂	152.0 (C2); 151.1 (C3); 149.7 (C5); 140.9 (C6); 43.3 (CH ₂); 28.5 (CH); 22.53 (CH(CH ₃) ₂), 21.8 (C3-CH ₃); 21.0 (C5-CH ₃)
 3,6-Dimethyl-2-isobutyl-	8.148, brs, 1 H, H5; 2.660, d, 7.3, 2 H, CH ₂ ; 2.521, d, 0.5, 3 H, C3-CH ₃ ; 2.491, d/q, 0.6/0.6, 3 H, C6-CH ₃ ; 2.130, mc, 1 H; CH; 0.951, d, 6.6, 6 H, CH(CH ₃) ₂	154.2 (C2); 150.0 (C6); 148.9 (C3); 140.6 (C5); 43.6 (CH ₂); 28.6 (CH); 22.50 (CH(CH ₃) ₂), 21.4 (C3-CH ₃); 21.1 (C6-CH ₃)

Table V. ^{15}N NMR Data for Both Isomers

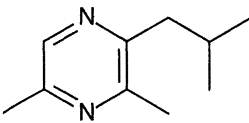
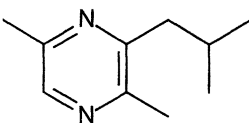
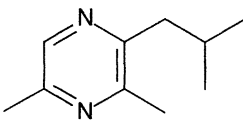
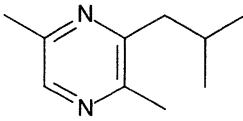
PYRAZINE	^{15}N Chemical Shift δ [ppm], position
 3,5-Dimethyl-2-isobutyl-	-44.8 (N1)
	-46.8 (N4)
 3,6-Dimethyl-2-isobutyl-	-45.1 (N1)
	-46.0 (N4)

Table VI. ^1H , ^{15}N HMBC Correlations for Both Isomers

PYRAZINE	^1H , ^{15}N HMBC CORRELATIONS (chemical shifts δ in ppm)
 3,5-Dimethyl-2-isobutyl-	N (-44.8) \leftrightarrow H (8.211); CH_2 (2.660)
	N (-46.8) \leftrightarrow CH_3 (2.532); CH_3 (2.488)
 3,6-Dimethyl-2-isobutyl-	N (-45.1) \leftrightarrow CH_2 (2.660); CH_3 (2.491)
	N (-46.0) \leftrightarrow H (8.148); CH_3 (2.521)

References

1. L.M. Nijssen, C.A. Visscher, H. Maarse, L.C. Willemsens and M.H. Boelens. *TNO Nutrition and Food Research Inst., Zeist, The Netherlands*, 7th Edition., 1996.
2. A. Arnoldi, C. Arnoldi, O. Baldi and A. Griffini, *J. Agric. Food Chem.* **1988**, *36*, 988
3. P.G. Keeney, *J. Am. Oil Chem. Soc.* **1972**, *49*, 567
4. M.J. Oruna-Concha, S. Craig Duckham and J. M. Ames. *J. Agric. Food Chem.* **2001**, *49*, 2414
5. W.M. Welty, R.T. Marshall and I.U. Grun, *J. Dairy Sci.* **2001**, *84*, 21
6. C. Counet, D. Callemien, C. Ouwerx and S. Collin, *J. Agric. Food Chem* **2002**, *50*, 2385
7. C.E. Mea., *Zhongguo Nongye Huaxue Huizhi* **1994**, *32*, 220
8. T.K. Chen, EP 590296, assigned to Societe Des Produits Nestle S.A., 1993.
9. R. Wagner, M. Czerny, J. Bielohradski and W. Grosch, *Z Lebensm Unters Forsch A*, **1999**, *208*, 308
10. The table is taken from FEMA guidance lines for application to the FEMA Expert Panel for Consideration of GRAS Status.
11. H. Sommer, H. J. Bertram, G. E. Krammer, C. O. Schmidt, W. Stumpe, P. Werkhoff and M. Zviely, *Magnetic Resonance in Chemistry* **2000**, *38*, 907
12. M. Zviely, A. Kern, I. Gozlan and R. Frim, *Flavours and Fragrances*, Ed. by K.A.D Swift, published by The Royal Society of Chemistry, 1997.
13. M. Zviely, A. Kern, I. Gozlan and R. Frim, *Perfumer & Flavorist* **1998**, *23*, 27

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