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Fruit Flavors

Biogenesis, Characterization, and Authentication

Russell L. Rouseff, EDITOR
University of Florida

Margaret M. Leahy, EDITOR
Ocean Spray Cranberries, Inc.

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M. Joan Comstock
Series Editor

Preface

FRUIT FLAVORS ARE WIDELY ACCEPTED AND almost universally esteemed. The floral, fruity flavors of many fruits combine gustatory pleasure with the promise of good nutrition and health. They have been used in a wide variety of highly successful juice beverages, syrups, and flavorings. Recently, unique flavor combinations of various fruit juice blends have been introduced in the marketplace with excellent consumer acceptance. New products incorporating fruit flavors have been developed. Applications range from fruit-flavored, ready-to-eat cereals to the recent introduction of fruit flavors into more traditional beverages such as tea. Fruit-flavored teas and lemonades are emerging successes that are forming a solid and substantial new niche in the beverage market.

With commercial success has come the need to better understand fruit flavors. Many of the key flavor-impact compounds for each fruit flavor have been determined, yet there are knowledge gaps. Some of the more exotic fruit flavors are still not well understood. The stability of key aroma-impact components during and after processing as well as their interaction with packaging materials are areas in need of further research.

The symposium upon which this book is structured was created to provide an overview of recent advances and developments in fruit flavors. New analytical and sensory techniques continue to be employed to better characterize fruit flavors. Many fruit flavors are marketed as natural flavors, and so methods to determine if the source of these flavors is of plant or synthetic origin must be developed. Biotechnology has been employed to generate key aroma compounds found in fruit flavors. Recent advances in the area of flavor precursors are reported. This is a relatively new area of research because it was only fairly recently discovered that many key aroma compounds in fruit flavors are stored naturally as glycosides. These flavor compounds are later released as the glycoside is cleaved from enzymatic or chemical action during processing or storage. These findings have in turn stimulated considerable study in fruit flavor biogenesis and the means by which variety, maturity, and horticulture practices may affect fruit flavors. All these areas are addressed in this book.

The information in this text would be of interest to anyone in the flavor industry or the food–beverage industry. Chemists would be interested in the analytical techniques used to determine flavor compounds. The information about flavor authenticity would be of especial interest to

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regulatory and quality control managers in the food and beverage industry. Creative flavor chemists and sensory and product development scientists would be interested in the basic characterization of fruit flavor, analytical and sensory correlation studies, biogenesis, and packaging effects. The information from this book was gathered from some of the world's most outstanding scientists actively working on fruit flavors.

Acknowledgments

We gratefully acknowledge with sincere appreciation the financial support for the symposium provided by Ocean Spray Cranberries, Inc., and the Division of Agricultural and Food Chemistry of the American Chemical Society. Without their support it would not have been possible to assemble the number of distinguished overseas scientists who contributed to this book. Finally, we express our appreciation to all of the authors, both for their excellent symposium presentations and their high-quality written manuscripts.

RUSSELL L. ROUSEFF
University of Florida
Citrus Research and Education Center
700 Experimental Station Road
Lake Alfred, FL 33850

MARGARET M. LEAHY
Ocean Spray Cranberries, Inc.
One Ocean Spray Drive
Lakeville-Middleboro, MA 02349

February 23, 1995

Chapter 1

Advances in Fruit Flavor Research

An Overview

Russell L. Rouseff[1] and Margaret M. Leahy[2]

[1]University of Florida, Citrus Research and Education Center, 700 Experimental Station Road, Lake Alfred, FL 33850
[2]Ocean Spray Cranberries, Inc., One Ocean Spray Drive, Lakeville-Middleboro, MA 02349

Fruit flavors continue to be of interest commercially and scientifically for a variety of reasons. Recent developments in fruit flavors are presented in this overview chapter. Major topics include: analytical chemistry of fruit flavors, correlating analytical and sensory measurements, flavor stability, packaging and storage interactions, flavor biogenesis, flavor precursors and biotechnology, authentication of natural fruit flavors, and fruit flavor varietal studies.

Fruits and vegetables are currently receiving considerable attention as consumers become more health conscious. Contemporary dietary recommendations are encouraging increased consumption in both these food categories. In this environment more and more fruit-containing foods will be developed and marketed to take advantage of the perceived healthfulness and the intrinsic preference for fruit flavors. A major factor to successfully increase consumption of fruits is delivering products with flavors perceived favorably by consumers. Many factors need to be considered to provide foods that will deliver high quality fruit flavors that persist after processing and storage.

Several areas impact the ability to successfully characterize the key components of fruit flavors. Advances in the analysis of flavors in general can be applied to fruit flavor research. Other key areas include correlating analytical and sensory measurements as well as flavor packaging and storage interactions. Marketers find the use of all natural flavors as advantageous, so methods to authenticate fruit flavors as natural are important for legal and ethical reasons. Because specific fruit cultivars can deliver very different flavor characters, research in the characterization of differences in flavor volatiles among cultivars is conducted. The area which has received the most recent interest is fruit flavor biogenesis and characterization of precursors. This is probably the fastest growing area of flavor research world wide.

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Analytical Chemistry of Fruit Flavors

Since fruit flavors exist as complex chemical mixtures, analysis has primarily involved separation technologies. Fruit flavors involve both volatile and nonvolatile flavor components. Sweet and sour are the major nonvolatile flavors in fruit and HPLC has been the method of choice to determine the corresponding individual sugars and acids. Although new variations continue to be published, HPLC methods to determine sugars (using either electrochemical or refractive index detection) or acids using UV detection have been known for some time. Bitterness is a flavor factor sometimes found in fruit. Methods to quantify and remove these bitter compounds from fruit products have been published (1). Multilayer coil countercurrent chromatography (MLCCC), a relatively new technique, has been used to isolate polar, thermally unstable flavor compounds such as ionone glycosides from quince fruit (2).

Major fruit flavor impact components are usually volatile and are typically determined using capillary gas chromatography, GC, GC-MS or GC-FTIR. Recent advances in this area include multidimensional GC, where the second column is a chiral column. This technique has been used for the enantiodifferentiation of isomeric vitispiranes in quince juice (3).

An important factor in properly interpreting any chromatogram is to understand how the flavor volatiles were concentrated and introduced on the column. Today, steam distillation extraction is generally avoided as it know to produce thermal decomposition artifacts. Current practices either extract the flavor components from the liquid or solid with a low boiling liquid, supercritical fluid or distill the volatile components under vacuum. Another possibility is to work with headspace gases and trap their volatiles either cryogenically or on a solid phase absorbent. The use of solid phase micro-extraction with subsequent thermal desorption using fused silica fibers (4) is a recent innovation. This technique has been used to extract flavor volatiles in model flavor systems, as well as coffee, fruit juice and a butter flavored vegetable oil (5). Using cupuaçu, a relative of cocoa, Fischer and coworkers compare chromatographic results from solvent extraction, vacuum distillation and simultaneous distillation/ extraction in an analytical section chapter in this book. They clearly demonstrate that the composition of the extracts is dependent on the isolation procedures employed. There are numerous variations to sample concentration but their complete discussion is beyond the scope of this work.

Combining Analytical and Sensory Measurements

The volatile composition of many fruits is extremely complex. Gas chromatography coupled to mass spectrometry is used routinely by flavor chemists today to separate and identify these volatiles. Since many, if not most, of the volatile components are *not* flavor active, the more difficult problem is to interpret *which* combination of components in *what* proportion is responsible for the perceived aroma. Sensory scientists have become increasingly sophisticated in their ability to characterize flavors using trained human assessors. Several chapters in Williams and Atkins book (6) on sensory analysis are devoted to correlating sensory and instrumental (chemical) measurements. Subsequent publications have utilized a variety of experimental designs

and mathematical treatment of the data. Some workers have developed straight forward statistical correlations primarily based on linear regression (7) while others have used multivariate statistics to correlate the two sets of measurements (8-10).

Several variations to these approaches can be found in the literature. One approach utilizes multivariate statistics to calculate which chromatographic components are most highly correlated with the sensory data. This approach recognizes that while individual flavor components are responsible for taste and aroma, the total sensation determined from sensory analysis is a result of the integration of all the individual flavor stimuli in a mixture. The advantage of this approach is that it will model more accurately the synergistic and interactive nature of flavor and nonflavor active components as they produce the total sensory impression. The disadvantage of this approach is that some components many be chosen for the flavor model only because they were highly correlated but not causative agents. When this happens, any change in the sample set will produce a new set of components and the model will have to be changed. To eliminate this problem some workers have developed their models from only those compounds shown to be flavor active from GC-olfactory data. The strength of this approach is that it provides a direct sensoric determination as to whether a particular GC component has aroma activity. However, it does not address how the total aroma *mixture* interacts in the original product. Examples of this approach can be found in the chapters in this book by Fischer and coworkers as well as the chapter by Guichard among others.

The chapter by Burgard discusses different approaches to developing multivariate mathematical models to describe flavor systems and guide product development. Shaw and coworkers also employ one version of this approach in an earlier chapter on orange juice. Young considered both volatile and nonvolatile flavor components in the overall flavor perception of kiwifruit in his chapter. In another chapter, Noble and coworkers reviewed the sensory and chemical information of Cabernet Sauvignon wine flavor. Using isotopically labeled methoxypyrazine, they have been able to correlate horticultural variables such as vine vigor, light intensity and soil type, with wine flavor and the concentration of isobutyl methoxypyrazine.

Flavor Stability, Packaging and Storage Interactions

In commercial products, initial flavor has little meaning, if that same flavor is not present when the product is consumed. The flavor changes that occur between production and consumption are of enormous interest to the food and flavor industry. Many factors have to be considered. Unfortunately many flavor active components are also highly chemically reactive and will react with each other, other product components or with the packaging they come in contact with. Because of their commercial importance, most of the work in this area has been done with apple or orange juice. Moshonas and Shaw (11) found that the hedonic flavor of commercial orange juice decreased most rapidly during the first week or two of storage. Higher storage temperatures produced the greatest decrease in flavor scores. During six weeks storage, they found increased levels of ethyl acetate which they speculated may have come from the laminated multilayered package liner.

Imai and coworkers (12) monitored the sorption of d-limonene, neral and geraial from orange juice into three sealant films during 24 days contact at 22 ^{o}C. They found

the co-polyester had less sorption of the organic volatiles than ethylene vinyl copolymer or commercial low density polyethylene. Konczal and coworkers (13) carried out a similar study with apple juice, however they used dynamic head space to determine sorption. Sorption of the apple juice volatiles was most significant for the low density polyethylene than with the two developmental polymers.

Sadler and coworkers discuss the interaction of orange juice with various packaging polymers in a chapter in this book. They found that d-limonene absorption increased microbial proliferation and accelerated vitamin C degradation. They were able to calculate the time required for identifiable flavor loss using diffusion, solubility and permeation data. Two inexpensive methods for evaluating the interaction between volatile organic compounds and packaging polymers were also presented.

Understanding Fruit Flavors - Biogenesis, Flavor Precursors and Biotechnology

The most rapidly growing area in fruit flavor research is focused on understanding the biogenesis of flavor compounds and characterization of precursors. Schreier and Winterhalter (14)published conference proceedings on progress in flavor precursor studies. This work included papers on the role of glycosidic precursors in flavor generation in wine, fruit and juices.

Biogenesis of grape and wine volatiles has received considerable attention. Sefton et al. (15) studied the flavor precursors of Chardonnay juices, analyzing for the free and glycosidically bound volatiles. Allen (16) investigated the viticultural effects on methoxypyrazine grape flavor. The significance of viticultural and enological practices on monoterpenes in *Vitis vinifera* berries and juices from British Columbia was studied by Reynolds and Wardle (17). Shure and Acree (18) investigated changes in the odor-active compounds in *Vitis labruscana* cv. Concord during growth and development. Glycosidic precursors of varietal grape and wine flavor were studied by Williams et al. (19). The effects of cloning and pruning on terpene yields in Muscat were investigated by McCarthy (20). Skouroumounis et al. (21) studied the precursors of damascenone in fruit juices. Damascenone is thought to be an odor-active compound contributing to the flavor of grape and many other fruit juices.

The biogenesis of flavor volatiles and post-harvest changes in other fruits is also an active area of research. Schreier's and Winterhalter's groups have conducted significant research in the biogenesis of flavor volatiles in exotic fruits such as lulo (22), starfruit (23), quince (24), and passion fruit (23). Prabha et al. (25) has investigated flavor formation in callus cultures of guava. Dettweiler et al. (26) studied the occurrence of C-8 diols and mechanism of formation in apples, and the relation of these diols to biosynthesis of other compounds of importance for apple. Free and glycosidically bound volatiles in hog plum were characterized by Adedeji et al (27). Post-harvest changes in flavor volatiles have been studied in passion fruit (28) and kiwi (7).

Authentication of Natural Fruit Flavors

Authentication of fruit flavors as natural is important in assuring compliance with labeling regulations. Methods of adulteration become sophisticated in response to developing methodologies for detection. Significant research has been conducted to detect adulteration in citrus essential oils (29-32). Methods of detection of adulteration

include analyzing ratios of key terpene components, ratios of enantiomeric compounds such as linalool and nonvolatile components such as coumarin. Analysis for the presence of flavor solvents is also a useful tool for detecting adulteration of citrus and other natural fruit essences, oils and extractives.

The use of enantiomeric ratios has been investigated for establishing the origin of several volatiles in fruit flavor (3;33-35). The enantiomeric composition of lactones in several fruits has been established using multidimensional GC/MS. While these techniques may be useful in identifying adulteration in single fruit flavors, it must be remembered that other enantiomeric ratios may exist when these compounds are produced in other natural systems, such as via fermentation. The use of deuterium and C13/C12 isotopic ratios has allowed differentiation of natural and synthetic benzaldehyde (36) and terpenoid flavor compounds (37).

Flavor Varietal Studies

Because specific fruit cultivars can deliver very different flavor characters, research in the characterization of differences in flavor volatiles among cultivars has been conducted. Many of these investigations focus on correlating analytical and sensory data. Two recent studies focused on flavor differences among apricot cultivars (38-39). A study focusing on 5 raspberry cultivars (40) was unable to establish a relationship between sensory and chemical volatile data.

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Chapter 2

Analytical Investigation of the Flavor of Cupuaçu (*Theobroma gradiflorum* Spreng.)

Norbert Fischer, Franz-Josef Hammerschmidt, and Ernst-Joachim Brunke

DRAGOCO AG, Research Department, D-37601 Holzminden, Germany

The Cupuaçu (*Theobroma grandiflorum* Spreng.) tree, a relative of cocoa (*Theobroma cacao* L.), is indigenous to Amazonia, Brazil. The pulp of its fruits is consumed e.g. in juices, ice creams or bakery fillings, especially in Brazilian Belem region. As a part of our ongoing project aimed at the investigation of less common tropical fruit flavors, the flavor of cupuaçu pulp was analyzed. Flavor extracts were prepared by using vacuum distillation, solid phase extraction and simultaneous steam distillation-extraction (SDE). The concentrates were evaluated sensorially, and analyzed by means of GC-, GC/MS- and GC-O techniques. Several major to minor components of sensory importance for the cupuaçu flavor were identified along with a number of trace constituents with very high flavor impact. The portion of short-chain acids, responsible for the typical acidic aspects of cupuaçu flavor, is mainly associated with the fibrous part of the pulp, whereas the distillate is dominated by several esters. 2-Ethyl-5-methyl- 4-hydroxy-3(2H)-furanone could be identified as an important trace component in the concentrate obtained by solid-phase extraction on RP-18 material. Thermal treatment of cupuaçu pulp produced additionally a bread-like flavor impression, for which 2-acetyl-1-pyrrolin was found to be responsible.

The flavor of exotic tropical fruits is of increasing interest for consumers throughout the world. Some tropical fruits, e.g. mango and passion fruit, already possess a great marketing potential, and, consequently, the demand for the respective flavorings increases. Lesser known fruit crops are either of regional commercial importance, or are appealing to flavorists and analytical chemists

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because their sensory properties. An internationally active flavor company like DRAGOCO has to address present and future needs of the customers worldwide. We therefore started an ongoing long-term R&D project aimed at the investigation of sensorially attractive tropical fruits of regional consumption from different regions of the world.

The cupuaçu tree (*Theobroma grandiflorum* Spreng.)is a relative of cacao (*Theobroma cacao* L.); its fruits are of obovoid shape and vary in weight from 200 -5000 g. Their hard, woody skin enclose the seeds and the edible part, the creamy-white to yellowish pulp. During an excursion through the Brazilian Manaus region, a DRAGOCO flavor expert experienced the highly desirable, intense, sweet-sour, exotic flavor of the cupuaçu fruit. It is consumed especially in the Manaus region as dessert together with cream and yoghurt, as juice or as bakery filling. The pulp is mainly consumed fresh, but also marketed as preserve in pasteurized form, sometimes with added sugar, or frozen *(1)*.

Literature data about the flavor compounds or the composition of volatiles of cupuaçu are rather scarce; one article about the volatiles of some Amazonian fruits *(2)* lists 11 components for the Likens-Nickerson-distillate of the canned fruit. In this paper we wish to report about our detailed analytical work on the flavor of this attractive tropical fruit.

Experimental

Fruit material. Cupuaçu pulp was prepared from fresh, intact fruits. The seeds removed, and the pulp immediately deep-frozen at the cultivation site in Manaus, Brazil. The frozen samples were transported via air-freight to DRAGOCO research department, Holzminden, Germany, and stored refrigerated until being further analyzed.

Vacuum distillation. "Cold" vacuum distillation of 1100 g pulp was performed as described recently *(3)* for approx. 4 hours; the distillate (230 ml) as well as the residue after distillation were extracted three times with diethyl ether.

Simultaneous steam-distillation-extraction (SDE). 300 g pulp were diluted with water and distilled in a SDE-apparatus *(4)*, using ether as an extraction solvent; small scale trials (30 g pulp) were performed after pH-adjustment to pH 3, 7 and 9 with appropriate buffer solutions.

Solid phase extraction. The clear juice obtained by centrifugation from 590 g pulp was passed through a RP-18 column (10 g LiChroprep RP-18, 40-63 μm). The column was washed with water, then eluted with ether and methanol. The ether extracts were used for the flavor analyses.

Isolation and derivatization of acids. Centrifugation of the pulp yielded the juice (see above) and pellets of gelatinous structure, mainly containing the fruit fibers. The pellets were washed with water, the water removed by suction using a filter funnel, and the solids were washed with ether and with methanol. The latter extracts were used for assaying the acid portion of flavor components. The acids in the methanol extract were derivatized with butanol-BF_3 to give the butyl esters according to *(5)*.

Handling of flavor extracts. All solvent extracts were dried over Na_2SO_4, and concentrated in a water bath (45° C) using a vigreux column to a volume of 0.5 - 1 ml; these concentrates were used for the GC analyses.

Gas Chromatography. 1. GC Shimadzu 14a, split/splitless injector, inlet split onto columns: a.) 60 m x 0.25 mm DB-1, film 0.25 μm b.) 60 m x 0.25 mm DB-Wax, film 0.25 μm, two FID, simultaneous registration of both channels and calculation of retention indices for both columns using integrator Shimadzu CR 4a;

2. GC HP 5890, on-column injector, column 30 m x 0.32 mm DB-1, 0.5 μm film, post-column split to FID, NPD and Tracor FPD; integrators Spectra-Physics SP 4290 and HP 3390 A.

Gas Chromatography-Olfactometry (GC-O). *Non-polar column*: 30 m x 0.32 mm DB-1, 1 μm film, temperature program 40°- 4°/min - 240°; gas chromatograph Siemens SiChromat 1-4, PTV-injector, post-column split to FID and heated glass sniffing port, additional supply of humidified purge air; integrator Spectra-Physics ChromJet. *Polar column*: 30 m x 0.32 mm DB-Wax, 0.5 μm film, temperature program 50°-3°/min-240°, gas chromatograph Packard 438, split / split-less injector Gerstel, Mühlheim / Germany, post-column split to FID and heated glass sniffing port, additional supply of humidified purge air; Integrator Shimadzu CR 3a.

Gas Chromatography-Mass Spectrometry (GC-MS). HP 5970 (MSD), ionization current 70 eV, ion source temperature 200°C; columns:

1. 60 m x 0.25 mm / 0.25 μm DB-1;
2. 60 m x 0.25 mm/ 0.25 μmDB-Wax; temperature program 40°-4°/min-240°.

Results and Discussion

In order to obtain flavor concentrates that are representative of intact fruit flavor, several different procedures for the flavor isolation were applied (see experimental). The flavor concentrates obtained were evaluated sensorially by our flavorists, and the best samples as well as those possessing characteristic aspects of the whole flavor profile, were analyzed by means of GC- and GC-MS-techniques. The sensory contribution of individual constituents was assayed by using the GC-O technique.

Vacuum distillation

The flavor extract obtained by cold vacuum distillation showed a very fresh, volatile, fruity-estery profile. However, some sensory aspects, referring to the flavor "body" and to the acidic character were under represented.

The composition of this extract together with the sensory impressions perceived during GC-O is listed in table I. Major sensory contributions in this extract are provoked by a variety of esters (e.g. ethyl hexanoate, ethyl butanoate, ethyl 2-methylbutanoate), by alcohols like linalool and 3-methyl butanol, and additionally by cis-3-hexenal and a further "green"-type component, which we have

not been able to identify up to now. These components, together with further less intense substances, are responsible for the fruity, apple-like aspects of cupuaçu flavor. The amount of volatiles obtained by this procedure (determined via internal standard tridecane) was calculated as approx. 3 ppm (no correction for individual response factors made).

Table I: Volatile components in the cold vacuum distillate from cupuaçu pulp

$I_{K\ DB\text{-}1}$[1]	substance	area-%	sensory description[2]	intensity[3]
	ethanol	ca.20	-	-
	ethyl acetate	10.5	-	-
569	diacetyl	0.5	butter-like, pungent	2
583	acetic acid	1.0	sour, acetic acid	2
649	butanol	1.9	-	-
666	2-pentanone	2.0	-	-
676	3-pentanone	0.1	-	-
679	2-hydroxy-3-butanone	0.3	-	-
684	2-pentanol	0.6	-	-
707	methyl butanoate	2.6	sweaty	1
716	2,4,5-trimethyl-1,3-dioxolane	0.4	-	-
721	3-methyl butanol	1.8	chocolate-like	3
725	2-methyl butanol	0.5	-	-
745	methyl crotonate	10.6	sweaty, butter	1
757	toluene	2.5	-	-
776	butanoic acid	tr.	sweaty	1
778	*cis*-3-hexenal	0.1	grassy, green	3
786	ethyl butanoate	2.2	ester-like, sweet	2-3
826	ethyl crotonate	4.5	-	-
839	ethyl 2-methylbutanoate	0.1	sweet, fruity, apple	2-3
842	*cis*-3-hexenol	0.9	grassy, green, sweet	2
850	methyl tiglate	tr.	-	-
857	hexanol	1.8	fatty	1
871	2-heptanone	0.1	-	-
883	methyl 2-hydroxy-3-methyl butanoate	0.1	-	-
907	methyl hexanoate	2.0	-	-
912	methyl *cis*-3-hexenoate	0.1	-	-

[1] Kovats index on DB-1
[2] description of sensory impression during GC-O
[3] intensity scale: 1 = weak, 4 = strong

Continued next page

Table I: Volatile components in the cold vacuum distillate from cupuaçu pulp *(continued)*

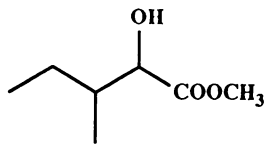
$I_{K\ DB\text{-}1}$[1]	substance	area-%	sensory description[2]	intensity[3]
948	methyl *trans*-2-hexenoate	14.0	green, herbaceous	1-2
961	unidentified	tr.	green, chemical, pungent	3-4
971	hexanoic acid	1.0	-	-
974	methyl 2-hydroxy-4-methyl-pentanoate	0.5	-	-
976	methyl 2-hydroxy-3-methyl-pentanoate	0.5	-	-
983	ethyl hexanoate	0.9	sweet, esterlike, winey	3-4
987	ethyl *cis*-3-hexenoate	0.1	-	-
990	ethyl *trans,trans*-2,4-hexadienoate	0.1	-	-
997	*trans*-2-hexenoic acid	tr.	-	-
1000	4-methyl-5-vinyl-thiazol	tr.	-	-
1004	gamma-hexalactone	tr.	-	-
1024	ethyl *trans*-2-hexenoate	1.5	sweet, fruity	2
1029	methyl 3-hydroxyhexanoate	0.1	-	-
1048	dimethyl succinate	tr.	-	-
1057	octanol	tr.	-	-
1087	linalool	1.1		
	+ 2-phenyl ethanol	0.1	sweet, floral, linalool	3-4
1106	methyl nicotinate	0.2	dry, powdery, pungent	1-2
1129	unidentified	tr.	fatty, aldehydic, cucumber	1
1150	methyl *trans*-2-octenoate	0.2	-	-
1155	octanoic acid	tr.	-	-
1175	alpha-terpineol	tr.	-	-
1180	ethyl octanoate	tr.	-	-
1189	4-vinyl phenol	0.1	-	-
1203	3-phenyl propanol	0.1	-	-
1219	hexyl crotonate	tr.	-	-
1224	ethyl *trans*-2-octenoate	tr.	-	-
1352	methyl cinnamate	0.1	sweet, fruity, warm	1

[1] Kovats index on DB-1
[2] description of sensory impression during GC-O
[3] intensity scale: 1 = weak, 4 = strong

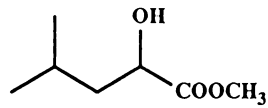
Ether extraction

The diethyl ether extract obtained from the residue after vacuum distillation, which covers additionally more polar and less volatile components, showed a qualitative composition very similar to the vacuum distillate. Some quite

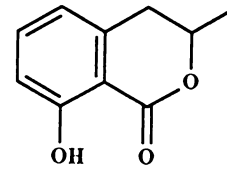
uncommon hydroxy esters like methyl 2-hydroxy-3- and 4-methyl- pentanoate (I) and (II) were present in higher amounts, as well as the isocoumarin-derivative (III). Compound (I) has not been described as a food constituent *(6)*, (II) was found once as a component of cape gooseberry *(7)*; the ethyl esters corresponding to (I) and (II) are present in a variety of alcoholic beverages *(8)*. 3,4-Dihydro-8-hydroxy-3-methyl-2(1H)-benzopyran-1-one (III) was found e. g. in soursop *(9)* and in guava *(10)*, both also tropical fruits. The sensory contribution of the components above is negligible, at least in GC-O.



(I)



(II)



(III)

The major portion of the ether extract consisted of higher fatty acids like myristic, palmitic, oleic, linoleic and linolenic acid (ca. 70 %). The total amount of volatiles as determined via GC (see above) was approx. 11 ppm. The odor contribution of the ether extract was quite weak, consequently it was not investigated further with regard to individual flavor components.

Localization of fruit acids

As already mentioned, both extracts obtained by vacuum distillation as well as by ether extraction did not sufficiently represent the typical fruity-acidic aspects of the cupuaçu flavor. A simple observation gave hints as to the localization of these flavor-relevant acids. Centrifugation of the cupuaçu pulp yielded the juice and a soft, slightly gelatinous pellet, consisting of the fibrous parts of the pulp.

These fibers released the typical acidic smell when rubbed between the fingers. We therefore concluded that the acid portion should be associated with the fibers. By washing the pellet with water or rinsing with ether (after removing the water by suction), the acids could not be dissolved; rinsing with methanol gave an extract which showed the typical fruity-acidic smell which was missing in the above-mentioned extracts. The acids in the methanol extract were derivatized to yield butyl esters *(5)* and analyzed by GC and GC-MS. The distribution of short-chain acids in this extract is shown in table II.

Main components are formic, acetic, 4-oxo-pentanoic, succinic, fumaric and malic acid; the odor contribution is probably mainly provoked by formic, acetic, propanoic, butanoic, and 2-methyl butanoic acid. Additionally some higher fatty acids were found in this extract (see table III).

Table II: Short-chain acids in the methanol extract obtained from the fibrous portion of cupuaçu pulp (Determined as butyl esters after derivatization with BF3 / butanol)

acid	relative amount
formic	+++
acetic	+++
propanoic	+
butanoic	+
lactic	+
2-methyl butanoic	+
pentanoic	+
angelic (*cis*-2-methyl-2-butenoic)	+
laevulinic (4-oxo-pentanoic)	+++
succinic (butanedioic)	++
fumaric (butenedioic)	++
malic (2-hydroxy-butanedioic)	+++

+ < 1%
++ < 10%
+++ > 10%

Table III: Higher fatty acids in cupuacu

acid	relative amount
myristic	+
palmitic	+++
heptadecanoic	+
oleic	+
stearic	+
linoleic	+
linolenic	++

+ <1%
++ <10%
+++ > 10%

Solid phase extraction

The cupuaçu juice obtained after centrifugation and removal of the fibrous portion was used for further investigations. Solid phase adsorption on non-polar stationary phases (e.g. RP-18, XAD-2, see experimental) is a well-established method to enrich free flavor components, including more polar molecules which tend to escape distillation and solvent extraction techniques, as well as the so-called

bound portion of flavor components, comprising e.g. glycosidically bound forms. The free flavor can be obtained by eluting the column with low to medium polar solvents like pentane, dichloromethane or ether, the bound portion is eluted by ethyl acetate or methanol.

The ether eluate obtained from RP-18 adsorbed cupuaçu juice showed a very appealing, fruity-sweet flavor, which contained more of the fruit "body notes" compared to the vacuum distillate. The components in this extract as well as the sensory impressions registered during GC-O are shown in table IV. Strong flavor impressions were provoked by major to minor components like butanoic acid, cis-3-hexenal and cis-3-hexenol, ethyl hexanoate, methyl nicotinate and methyl 5-hydroxy-trans-2-hexenoate. The latter component has not yet been described as a food constituent; its tentative identification is based on the mass spectrum reported in *(11)*. Methyl nicotinate was found in fruits like papaya *(12)*, mountain papaya *(13)*, guava *(10)* and strawberry *(14)*.

Additionally, several trace constituents were recognized to deliver major contributions to the overall flavor profile by means of GC-O. Among these traces we could identify methional with its typical potato-like smell, 2,5-dimethyl-4-hydroxy-3(2H)-furanone, possessing a fruity caramel-like smell, reminiscent of strawberry, and vanillin, which even in trace concentrations provoked a very intense vanilla-like flavor impression. Furthermore, a very intense caramel-like smelling trace was recognized, immediately reminding of the well-known 2,5-dimethyl-4-hydroxy-3(2H)-furanone. The retention index of this more intense component was considerably higher. By comparison with an authentic reference compound, the unknown component could be identified as 2-ethyl-5-methyl-4-hydroxy-3(2H)-furanone (IV), the corresponding homologue. The identification is based on the sensory characteristics during GC-O and on the retention indices on DB-1 and DB-Wax columns. This hydroxyfuranone was described up to now only in coffee, soy sauce and in musk melon *(15-17)*, in contrast to the frequent occurrence of the dimethyl hydroxy-furanone (known as Furaneol^{R}) in food flavors.

Two further trace constituents could not be identified up to now; the first one, already mentioned above in the vacuum distillate, with an intense green-pungent and chemical odor, and a retention index of 961-963 on DB-1 and of 1378-1380 on DB-Wax, was again one of the most important flavor components in the RP-18 isolated extract. The second one, with even lower concentration, possessed a very intense, characteristic sulphurous, black currant-like smell and retention indices of 1120 on DB-1 and 1596 on DB-Wax. Of both components no mass spectrum could be obtained, and the retention data are not in agreement with known components of similar flavor characteristics.

A reexamination of several solvent extracts obtained from cupuaçu on the basis of the retention indices of the above mentioned important flavor components in the RP-18 isolate confirmed the presence of all of them in the extracts also.

Table IV: Flavor components in the ether eluate from RP-18 adsorbed cupuaçu juice

$I_{K\ DB\text{-}1}$[1]	substance	area-%	sensory description[2]	intensity[3]
583	acetic acid	4.3	acidic, pungent	2
606	acetaldehyde ethyl methyl acetal	0.3	-	-
620	butanol	1.7	-	-
665	2-pentanone	1.7	-	-
679	3-hydroxy-2-butanone	0.4	-	-
684	2-pentanol	0.9	-	-
707	methyl butanoate	4.1	sweet, fruity	1
716	2,4,5-trimethyl-1,3-dioxolane	0.3	-	-
721	3-methyl butanol	1.4	chocolate-like	1-2
725	2-methyl butanol	0.3	-	-
744	methyl crotonate	14.9	-	-
757	toluene	0.9	-	-
775	butanoic acid	1.4	sweaty, butyric acid	3-4
779	*cis*-3-hexenal	0.1	green, grassy	2-3
786	ethyl butanoate	1.8	sweet, ester-like	1-2
825	ethyl crotonate	1.7	-	-
829	*trans*-2-hexenal	0.1	-	-
839	ethyl 2-methyl butanoate	0.1	sweet, apple, ester	2
842	*cis*-3-hexenol	1.6	green, grassy, apple	2-3
856	methyl 3-hydroxy-3-methyl butanoate + hexanol	2.5	fruity, sour, fresh	2
868	methional	tr.	potato, methional	2-3
871	2-heptanone	0.1	-	-
884	methyl 2-hydroxy-3-methyl butanoate	tr.	-	-
908	methyl hexanoate	2.3	-	-
913	methyl *cis*-3-hexenoate	0.2	-	-
931	benzaldehyde	tr.	-	-
947	methyl *trans*-2-hexenoate	16.0	ester-like, herbaceous	1-2
963	unidentified	tr.	green, acrid, metallic	4
972	hexanoic acid	5.5	sweaty, buttery	2
974	methyl 2-hydroxy-4-methyl pentanoate	2.1	-	-
977	methyl 2-hydroxy-3-methyl pentanoate	0.6	-	-
983	ethyl hexanoate	0.6	fruity, wine-like, sl. sweaty	3
987	ethyl *cis*-3-hexenoate	tr.	-	-

Table IV: Flavor components in the ether eluate from RP-18 adsorbed cupuaçu juice *(continued)*

$I_{K\ DB\text{-}1}$[1]	substance	area-%	sensory description[2]	intensity[3]
990	methyl *trans,trans*-2,4-hexadienoate	0.1	-	-
996	*trans*-2-hexenoic acid	0.6	-	-
1000	4-methyl-5-vinylthiazol	0.2	-	-
1005	gamma-hexalactone	0.4	-	-
1023	ethyl *trans*-2-hexenoate	0.4	-	-
1029	2,5-dimethyl-4-hydroxy-3(2H)-furanone	tr.	sweet, caramel, strawberry	1-2
1030	methyl 3-hydroxy-hexanoate	0.5	-	-
1043	methyl 3-oxohexanoate	0.3	-	-
1049	delta-hexalactone	0.2	-	-
1057	methyl 5-oxohexanoate	0.1	musty, rubber-like	1-2
1061	*cis*-linalooloxide (fur.)	0.2	floral, earthy, sweet	2
1075	*trans*-linalooloxide (fur.)	0.2	sweet, herbaceous	2
1082	methyl 3-hydroxy-2-methyl pentanoate	tr.	-	-
1087	linalool	1.0		
	+ 2-phenyl ethanol	0.2	sweet, floral, linalool	3-4
1100	methyl 5-hydroxy-hexanoate	tr.	-	-
1104	methyl nicotinate	1.9	dry, powdery, sweet	3
1105	2-ethyl-5-methyl-4-hydroxy-3(2H)-furanone	tr.	caramel, strawberry	4
1119	unidentified	tr.	sulphurous, blackcurrant	4
1129	methyl 5-hydroxy-*trans*-2-hexenoate	1.8	sweet, fruity	2-3
1150	benzoic acid	0.1	-	-
1155	octanoic acid	0.1	-	-
1193	4-vinyl phenol	0.7	-	-
1203	3-phenylpropanol	0.3	-	-
1213	gamma-octalactone + anisaldehyde	tr.	fatty, herbaceous + anise-like	2
1241	delta-octalactone	0.1	herbaceous	2
1251	nonanoic acid	tr.	-	-
1286	4-vinyl guaiacol	tr.	-	-
1336	4-allyl phenol	0.1	-	-
1354	methyl cinnamate	0.1	floral, sweet, fruity	1-2
1359	vanillin	tr.	vanilla-like	3-4
1370	unidentified	tr.	lactone-like, sweet	1
1397	unidentified	tr.	herbaceous, lavender	2-3

[1] Kovats index on DB-1
[2] description of sensory impression during GC-O
[3] intensity scale: 1 = weak, 4 = strong

However, their concentrations and consequently their sensory importance, were considerably smaller. This observation underlines the benefits of using solid-phase extraction as one additional method for flavor isolation, since components of higher polarity and low volatility can be obtained in better yields than with other methods.

Simultaneous steam-distillation-extraction

For comparison purposes a conventional steam distillation at atmospheric pressure [SDE, simultaneous steam-distillation-extraction *(4)*] was also used for the isolation of the flavor components of cupuaçu pulp. The concentrate obtained this way showed typical "cooked" flavor characteristics together with fruity notes, and, surprisingly, an additional strong bread-like flavor impression. GC-O on non-polar and polar columns led to the assumption that 2-acetyl-1-pyrrolin, the impact component of bread flavor *(18)* and of cooked rice *(19)* could be responsible for this flavor impression. A mass-spectrum, however, could not be obtained since the concentration of the flavor compound again was too low.

In order to increase the concentration of this compound, we repeated the SDE procedure with small samples of cupuaçu pulp at different pH-values. The sensory evaluation of these samples showed a considerable increase of the "bread"-note at pH 9. Repetition of this treatment with a higher amount of pulp (ca. 700 g) enabled obtain a concentration sufficient for recording a clean mass spectrum of the component being looked for (see fig. 1).

The identity of the bread-smelling component in this extract and of 2-acetyl-1-pyrrolin could be proven this way. The mechanism of the formation of 2-acetyl-1-pyrrolin and its possible precursor in cupuaçu however remain still unknown.

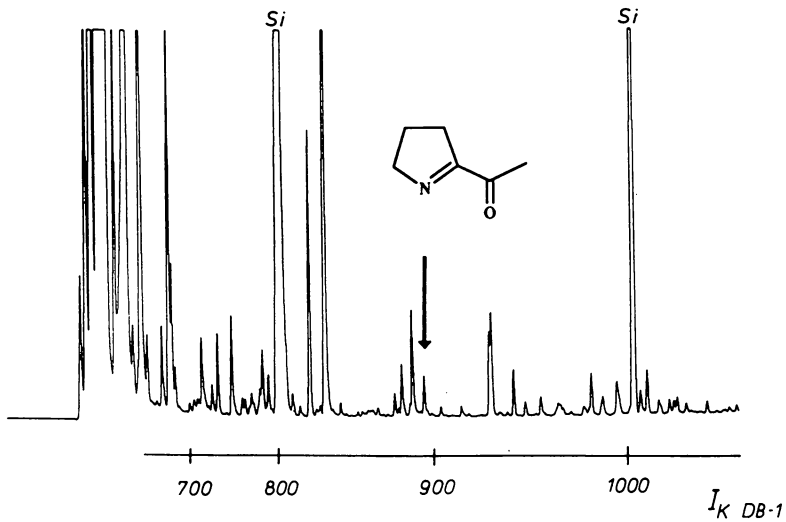


Figure 1: Chromatogram of the SDE-extract obtained at pH 9

Conclusion

Our analytical work on cupuaçu flavor resulted in the identification of several components, which are of major sensory importance for the overall flavor profile. The more fresh, volatile portion was represented in the vacuum distillate, the heavier fruity notes of higher polarity could be enriched in the extract obtained by solid-phase-extraction on RP-18 material. The investigations will be continued and shall lead to the formulation of a cupuaçu flavoring.

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Chapter 3

Chemical and Sensory Correlations for Orange Juice

David R. Burgard

Proctor and Gamble Company, Miami Valley Laboratories, P.O. Box 398707, Cincinnati, OH 45239

Concepts and methodology used in the study of multivariate relationships between chemical composition and sensory characteristics are discussed. An approach is demonstrated by analysis of a data set consisting of chemical and sensory measurements on orange juice. Variations caused by fruit variety, maturity, processing and product aging were present. Relationships between the chemical composition, expert panel sensory evaluations and consumer test scores were identified.

The ability to understand and model the effect of chemical composition changes on the sensory properties of food and beverage products is a subject of much interest(1-5). In *formulated* products, simple experimental designs can be used to identify *cause and effect* relationships. But the flavor in *natural* systems is a complex mixture that cannot always be studied by simple experimental designs. The large number of sensory active compounds make it difficult if not impossible to develop a model based on the psychometric functions of individual compounds.

There is an analogy between chemical data and the sensory scores obtained from a trained expert panel. As demonstrated in Figure 1, one would hope to be able to identify *cause and effect* relationships for chemical composition and *calibrated* human instruments. The situation becomes more complex when unknown *desirability or pleasantness* functions are imposed by consumers. This can result in a different set of *causes and effects*. To understand the effect of changes in chemical composition on product performance requires a translation of these different data types.

Multivariate analysis (MVA) techniques can be used to systematically organize and reduce large data sets. A multivariate analysis approach provides for simultaneous evaluation of all the data to identify trends among variables and relationships between samples. The principal components analysis (PCA) technique identifies correlated variables (eg. sensory attributes or chemical

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compounds), reduces the number of dimensions necessary to describe the data and allows similarities and differences between samples to be found by projection of the samples onto the newly defined PC axes.

Experimental

Chemical and expert panel sensory data were gathered to characterize not from concentrate (NFC) and chilled orange juice (COJ) products. Test NFC products made over a period of 4 months were compared to identify the effects of fruit variety and maturity. Aging studies were performed to compare commercially available NFC (CNFC) juice to the test NFC (CHNFC) products and to single strength chilled orange juice (CHCOJ). See Table 1 for a description of the characterization data.

TABLE 1. CHEMICAL AND SENSORY CHARACTERIZATION DATA

Chemical Compounds

Oil Derived Compounds	Essence Compounds	Sesquiterpenes
limonene, α-pinene,	acetaldehyde	valencene
ß-myrcene, ß-pinene,	ethyl butyrate	ß-caryophyllene
linalool, α-terpineol,	ethyl caproate	nootkatone
hexanal,	ethyl 3-OH hexanoate	
octanal, t-2 hexenal,	ethyl acetate	
nonanal, sabinene,	ethanol	
decanal, neral,		
dodecanal, 4-terpineol,		
c-3-hexenol, d-carvone		

Other chemical measures used were the brix/acid ratio (B/A).

Expert Panel Descriptors	Consumer Descriptors
sweet	sweetness
sour	tartness
bitter	bitterness
artificial	aftertaste
cooked	texture
natural	overall rating
fresh	fresh orange flavor
peely	

Sensory data were gathered by an expert panel consisting of at least 6 judges. Samples were rated on a scale of 0-10 compared to an FCOJ reference sample that had predefined values of the following attributes: sweet (5.5), sour (3.0), bitter (2.0), natural (4.0), fresh (4.0), artificial (2.5), cooked (2.5), fermented (0.0), and

peely (2.5). Since most of the samples were not fermented, this attribute was eliminated prior to data analyses.

Chemical analyses were performed on the same day as the sensory testing when possible. Volatile aroma and flavor compounds were monitored by a purge and trap headspace and extraction gas chromatography procedures. Results were reported in parts per billion (ppb). The brix and titratable acidity were measured by standard procedures.

For the aging studies, the juices were stored at 34°F and sensory evaluations were performed weekly for 10 weeks. Chemical analyses were done at 0, 1, 3, 5, 7, and 9 weeks. The data reported here are for weeks 0, 3 and 7 only, to reduce the clutter in the scatter plots. The same trends were present in the complete data set.

For a limited set of 9 samples, consumer taste tests were performed. Data were gathered on 3 samples each of CHNFC (from early Valencia, mid Valencia and late Valencia oranges), CHCOJ and CNFC juices. The tests consisted of single product taste tests on juices that were 2 weeks old. The numerical values used for comparison were average values derived from 5 point category rating scales. The overall rating scale was 0 = poor, 25 = fair, 50 = good, 75 = very good, 100 = excellent. The categories for the direct questions were converted as follows: "A lot less than I like" = 0, "A little less than I like" = 25, "Just the right amount" = 50, "A little more than I like" = 75 and " A lot more than I like" = 100.

The data were put into Lotus 123 files and analyzed using the Einsight or Pirouette software packages. Data were preprocessed by autoscaling prior to PCA. Initially, the chemical, sensory and consumer data were analyzed separately. Data sets were then combined for joint analyses. Trends and correlations identified were consistent throughout all analyses.

Results

Variations in NFC Production. The results of a joint PCA of the chemical and expert panel sensory data for the test NFC production runs are shown in Figures 2 and 3. Several groups of samples are visible in Figure 2. Products are coded by the production date and whether they were before (BP) or after (AP) pasteurization . Those is the lower left quadrant were produced on 2-06 from early-mid fruit. Those on the right hand side of the plot were produced on 2-27, 3-06, 3-20 and 4-01 from early Valencia fruit. Samples in the top center of the plot are from mid Valencia fruit. Those in the upper left were produced from late Valencia oranges on 5-21. This plot shows changes in the NFC product caused by the variety and maturity of fruit used.

Figure 3 allows us to see the *relative* compositional and sensory characteristics of the samples. For the purposes of discussion, the compounds are divided into 4 groups. The first group contains mostly the lighter essence compounds. Group 2 is the sesquiterpenes except for valencene. Group 3 compounds are primarily derived from the oil. Group 4 contains the remaining compounds. See Table 2

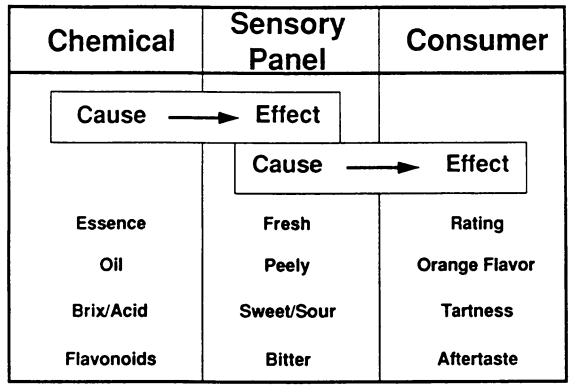

Figure 1. Demonstration of different sets of *cause and effect* relationships depending on the type of sensory data being analyzed.

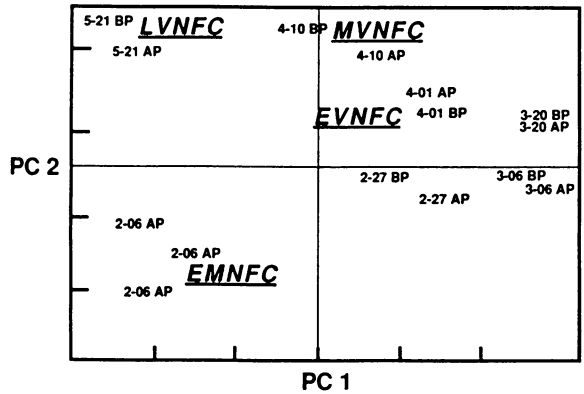

Figure 2. PCA scores plot showing the variations in NFC products across the fruit season.

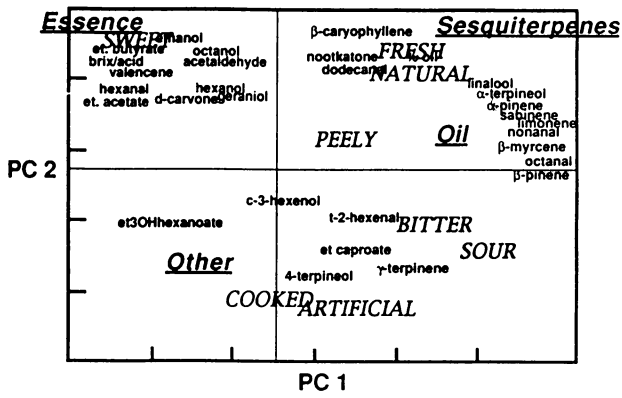

Figure 3. PCA loadings plot showing the relationships of the various groups of compounds to each other and to the expert panel product ratings.

for a detailed listing. The biggest changes in chemical composition for the production runs were in the levels of the oil, sesquiterpenes and essence and the brix / acid (B/A) ratio. Variations in the oil level are described by principal component #1. Variations in the essence and sesquiterpene levels are described by principal component #2. The relative levels of all the flavor chemicals varied during the fruit season. Comparison of Figures 2 and 3 shows that early in the season, they were all low. There was a large increase in the oil level with a small essence increase in early Valencia juice. Mid-Valencia juice differed from the early Valencia in that the oil levels were about the same but the essence and sesquiterpene levels were higher. The late Valencia juice had the highest B/A and essence levels with low oil levels.

Expert panel sensory ratings varied with the changes in composition as shown by the correlations in Figure 3. NFC juice from the early-mid fruit had low oil and low essence with relatively high levels of the C6 aldehydes. It was artificial, cooked, bitter and sour tasting with low fresh and natural ratings relative to the other NFC samples.

The early Valencia samples on the right side of Figure 2 are high oil, with moderate essence and sesquiterpene levels. These samples are more bitter, sour, artificial and cooked with low natural/fresh ratings relative to the later NFC samples. Within this group, the earlier samples (2/27, 3/06) were more cooked and artificial and less fresh/natural. The later samples (3/20 and especially 4/01) had better quality as a result of increasing essence and sesquiterpene levels with maturation of the fruit. All these samples had essentially constant high oil levels. The largest changes within this group were in the essence and sesquiterpene levels.

The mid Valencia samples were sweeter and had high fresh/natural ratings with low bitter/sour and artificial/cooked. They had high oil, high essence, high sesquiterpene levels and a higher B/A.

The late Valencia samples had low oil, high essence and high sesquiterpene levels. The high B/A made them the sweetest.

The average relative levels of the 4 different groups of compounds in each juice are summarized in Table 3. The data demonstrate the relationships discussed above.

Additional information was found in higher pricnipal components that showed the effects of pasteuriation. They were similar to those reported earlier (4).

NFC Aging Studies. Aging studies were performed on products made from early, mid and late Valencia fruit. CHCOJ and the CNFC samples were aged with the CHNFC products for comparison. The data from each study were analyzed separately and then combined. The trends were similar for the 3 different studies. The CNFC was consistently between CHNFC and CHCOJ. All products showed similar aging trends.

Figure 4 shows a comparison of the samples from the three aging studies. This plot shows both product compositional differences as well as changes with aging. CHCOJ and CNFC products are identified by the study number (S2,S3,S4), COJ or CNFC and the week (W0,W1,W3,W5,W7,W9) the tests were performed

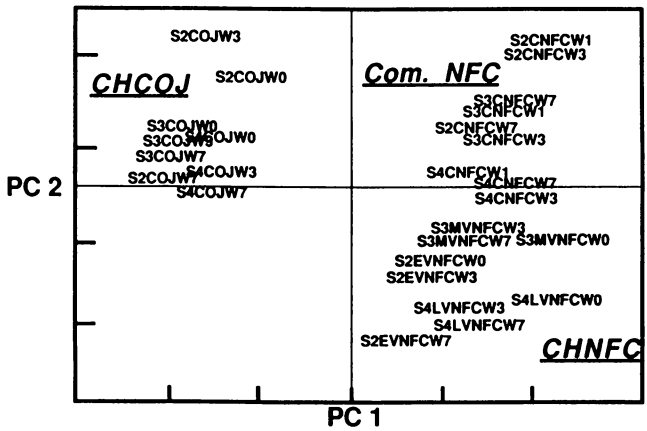

Figure 4. PCA scores showing the comparison of CHNFC, CHCOJ and CNFC in aging studies.

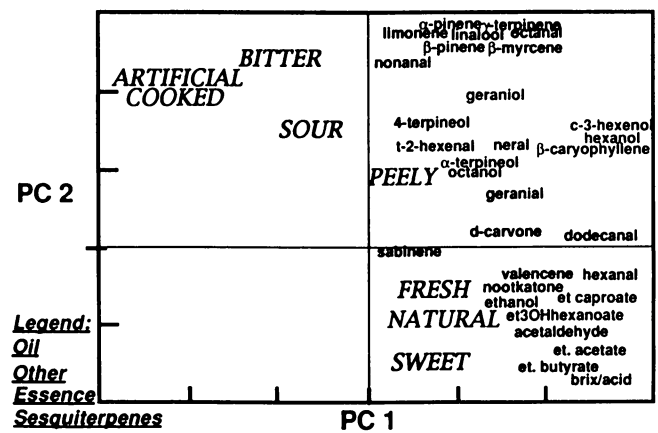

Figure 5. PCA loadings associated with the scores in Figure 4.

during the aging studies. NFC test products are identified by the study number, EV, MV, or LV for early Valencia, mid Valencia and late Valencia respectively and the week the tests were performed.

TABLE 2. GROUPS DEFINED BY PRINCIPAL COMPONENTS ANALYSIS

Correlated Group	Primary Source	Chemical Compounds	Sensory Attributes
1	essence	acetaldehyde ethanol, methanol ethylbutyrate ethylacetate, B/A octanol, hexanol hexanal, d-carvone geranial, valencene	sweet
2	sesquiterpenes	ß-caryophyllene, % oil nootkatone dodecanal	fresh, natural
3	peel oil	linalool, α-pinene, α-terpineol, neral sabinene, octanal limonene, nonanal ß-myrcene, decanal ß-pinene	peely
4	other	c-3-hexenol, hexanal ethyl-3OH-hexanoate t-2-hexenal, γ-terpinene ethylcaproate 4-terpineol	sour cooked artificial bitter

Figure 4 shows that each type of juice is compositionally different from the others. The variations shown in Figure 2 and discussed previously are small compared to the differences in the CHNFC, CHCOJ and CNFC samples. The CNFC samples are between the CHNFC and CHCOJ samples. The largest differences between the CHNFC, CHCOJ and CNFC were in the earlier studies. By the last study (S4COJ, S4CNFC and S4LVNFC) the differences between the products were smaller.

Figure 5 is the PCA loadings for the chemical and sensory data used to define Figure 4. Comparison to Figure 3 shows that the groupings of compounds and sensory scores are similar but not identical. In this data set, the biggest differences (principal component #1) were caused by the presence of the CHCOJ which was the most artificial/cooked, bitter and sour and had a high oil/low essence composition. The CHCOJ and CNFC samples are more bitter and sour than the CHNFC samples. The CNFC samples are more peely. CNFC has a higher oil to essence ratio and the highest absolute oil level.

TABLE 3. COMPARISON OF PCA GROUP AVERAGE LEVELS

JUICE	OIL	ESSENCE	SESQUITERPENE	OTHER
EM AVG	8146	455	138	339
EV AVG	20355	428	184	303
MV AVG	17752	541	255	309
LV AVG	14723	621	205	293
EM / EV	0.40	1.06	0.75	1.12
EM / MV	0.46	0.84	0.54	1.10
EM / LV	0.55	0.73	0.67	1.16
EV / MV	1.15	0.79	0.72	0.98
EV / LV	1.38	0.69	0.90	1.04
MV / LV	1.21	0.87	1.24	1.06

The second principal component shows that the CHNFC products were more sweet and fresh/natural with the highest essence levels. It splits the oil compounds into two subsets, indicating that different oil compositions are present in the three products or changes (eg. acid catalyzed hydrolysis) are occuring with time. Both situations are probably true .

All products show about the same aging trend, indicating that similar changes are occuring in each product. Aging is indicated by a loss of the oil and essence compounds and a decrease in the fresh/natural rating. The magnitude of change caused by aging was about the same as the differences within the same products across the three aging studies. The biggest product changes during aging were seen in the first aging study (S2) for the CHCOJ and CNFC. With the other products, changes with aging were less pronounced.

Chemical and Sensory Correlations. The PCA technique provides an indication of correlations between the measured variables. When chemical and sensory data are combined in an analysis, as was done here, correlations between chemical and sensory data can be explored. It must be noted that the presence of a correlation does not mean that a cause and effect relationship definitely exists. Rather, it means that both the variables are changing in a similar manner. There may be several reasons for such co-variation including *chance* correlations. All possibilities must be considered.

One can identify specific compounds that influence the sensory character by calculation of the ratio of the compound concentration to its sensory threshold (6). These ratios should only be used as an indication of the relative *order* of importance. Those below their sensory threshold (ratio < 1) do not have much sensory impact. Those near their threshold (ratio ≥ 1) are providing some of the basic flavor and those much above their threshold (ratio $>> 1$) yield the biggest sensory effect. It should be remembered that there are other compounds, whose levels are below the detection limits of the GC methods, that will effect the flavor because of their extremely low sensory thresholds.

Table 4 shows the ratio of juice levels to sensory thresholds for those compounds above their sensory threshold. They are organized into the essence, oil and sesquiterpene groups previously mentioned. Within each group, they are *ordered* from largest to smallest sensory impact. From this analysis, it appears that ethylbutyrate and ethylcaproate are the most important compounds in the essence fraction. Acetaldehyde and ethyl-3-OH-hexanoate are the next most important. For the oil fraction, octanal is giving the largest contribution followed by approximately equal contributions from the other aldehydes and terpenes. Note the large number of compounds nominally contained in the oil fraction that are of sensory significance. The only compound from the sesquiterpene fraction that shows some significance in this analysis is valencene.

The trends previously identified by PCA, combined with the threshold scaling approach provide a more complete picture about cause and effect relationships. The correlations used to define the groups in Figure 3, have practical significance when discussing the impact of flavor compounds. The levels of all of the compounds within a given group are varying simultaneously. That is, the levels of the essence compounds all increase or decrease simultaneously when comparing them across samples. The same is true for the compounds in the oil fraction and the sesquiterpenes. Thus, it is difficult to point to any one compound as providing important sensory impact in these groups. This is especially true for the oil fraction where most of the oil compounds are at about the same sensory significance. An exception is found in the essence fraction. Most of the "essence" derived impression comes from ethyl butyrate (due to its overwhelming sensory magnitude) with smaller contributions from the other compounds.

The PCA results also show that the brix/acid ratio (B/A) is correlated to the changes in the essence level. That is, as the essence level increased (later season fruit), so did the B/A. The effects of the two cannot be separated in this data set. This type of variable covariation is common in studies of natural products where one cannot independently vary individual parameters.

Comparison to Consumer Data. Results of consumer tests were compared to the expert panel (EP) sensory evaluations and chemical data for the same juices. A PCA analysis of the consumer and EP data on this selected subset of the data is shown in Figures 6 and 7. Consumer and expert panel data are printed in all capital letters. Expert panel scores begin with EP in these plots. Refer to Table 1 for attribute identifications. From this we see that numerous correlations exist both within the consumer and EP data sets and between them. The relationships among the EP sensory attributes are the same as those mentioned previously. Correlations within the consumer ratings will be discussed first.

Consumers' overall rating and fresh orange flavor rating are highly correlated. They seem to be driven primarily by texture and sweetness and are inversely related to tartness, aftertaste and bitterness. If the juice is too thin or not sweet enough, its overall rating will decrease. In this data set, no products were rated (on the average) as too thick or too sweet. Upper limits on these characteristics were not determined here. Conversely, a product that is too sour, too bitter or has too much aftertaste will receive a lower rating.

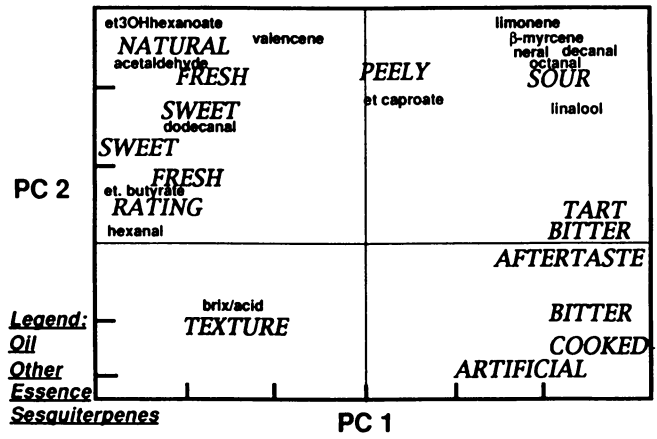

Figure 6. PCA loadings plot comparing compounds selected by threshold scaling, expert panel ratings and consumer ratings.

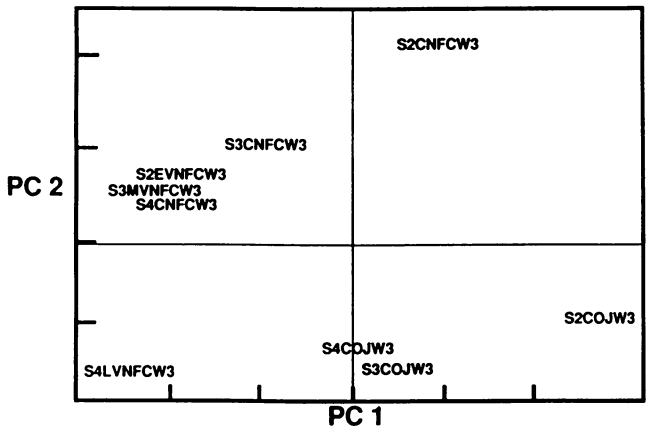

Figure 7. PCA scores plot comparing the samples for which consumer data were collected.

TABLE 4. MOST IMPORTANT FLAVOR AND AROMA COMPOUNDS AVERAGE LEVELS RELATIVE TO SENSORY THRESHOLD

Essence Compounds

JUICE	ethyl-butyrate	ethyl-hexanoate	hexanal	acetaldehyde	ethyl-3OH-hexanoate
CHNFC	5839	215	72	58	42
CHCOJ	2919	0	30	19	3
CNFC	4296	147	53	55	38

Oil Compounds

	octanal	β-myrcene	α-pinene	limonene	decanal
CHNFC	635	192	152	140	108
CHCOJ	895	242	226	171	175
CNFC	1070	308	236	237	216
	linalool	**nonanal**	**dodecanal**		**valencene**
CHNFC	98	58	70		161
CHCOJ	175	79	31		34
CNFC	203	102	78		161

Comparison of the consumer ratings to the EP attributes shows that no EP attributes are strongly correlated to the consumer fresh orange flavor or overall rating (Figure 6). This Figure also shows that the sweet perception of the EP is similar to the sweetness perceived by the consumer. Both suggest that "sweetness" is important to the consumer's rating. Since the brix/acid ratio naturally varied with the essence level (see discussion of chemical changes above), the relative importance of the effect of sugar solids and essence level changes cannot be separated by this data set. EP sour and consumer tartness are partially correlated to each other. The correlation between the ratings of bitterness and aftertaste suggests that aftertaste is bitter. The more easily recognized characteristics such as sweet, sour and bitter show the best correlations between the consumer and expert panel data. This is common for sensory evaluations of beverage products.

The less clearly defined EP attributes are not simply related to the consumer ratings. This is especially true for the consumers' fresh orange flavor and overall rating. Consumer fresh orange flavor/overall rating and EP fresh/natural are loaded (correlated) on PC#1, opposite of the negative attributes (artficial, cooked, bitter and sour/tart). EP fresh/natural are also strongly loaded on PC#2 but the consumer fresh orange flavor is not. This suggests that additional characteristics are being considered in the EP panel rating.

Conclusions

The large amount of data collected during the production and aging of the NFC products provided some useful insights. Variations in the CHNFC products caused by fruit variety were evident but generally smaller than the variations in the CHCOJ

and CNFC products tested during the same period of time. The CNFC had oil/essence ratios between CHNFC and CHCOJ. This was clearly seen in the chemical data and was probably responsible for the lower EP sensory ratings for CNFC. The major sensory discriminator of CHCOJ from the NFC products was the artificial/cooked note. The CNFC had slightly higher cooked scores than CHNFC contributing to its lower fresh/natural rating.

There were many co-variations in the chemical composition making it difficult to identify any one compound as being the most important. This is common for natural products. If one had to chose, the most important would be ethyl butyrate based on its level relative to its sensory threshold. There also was a partial inverse correlation between the oil land essence levels, making it difficult to entirely separate the effects of the two.

Acknowledgements

The contributions of the many other workers who planned and executed the production and testing of the products used in this work is gratefully acknowledged.

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Chapter 4

Multivariate Analysis for Classification of Commercial Orange Juice Products by Volatile Constituents Using Headspace Gas Chromatography

Philip E. Shaw[1], Manuel G. Moshonas[1], and Bela S. Buslig[2]

[1]U.S. Citrus and Subtropical Products Laboratory, South Atlantic Area, Agricultural Research Service, U.S. Department of Agriculture and [2]Florida Department of Citrus, 600 Avenue S, N.W., P.O. Box 1909, Winter Haven, FL 33882–1909

Analyses of fresh and processed orange juices by headspace gas chromatography afforded quantities of up to 40 volatile components in each juice type. Many of these components are known to influence citrus juice flavor. Multivariate analysis of the quantitative date with a computer pattern recognition program classified the various juice samples according to processing conditions. The graphically displayed classifications corresponded to expected flavor quality. These results can potentially help processors determine product quality without sensory evaluation measurements, and suggest changes in processing conditions to improve flavor of processed products.

The increasing availability of relatively inexpensive fast personal computers with increasingly large memory capacities has permitted development of many user-friendly multivariate analysis programs. These developments have opened up an important new area of data analysis in food chemistry, so that multiple analytical and/or sensory measurements on many samples of a food product can be easily compared, and separation of product samples into several groupings is often possible. Visual examination of the same data set is usually not adequate to distinguish the groupings which can be determined through multivariate analysis (1,2).

Orange juice and orange flavor fractions have been characterized by several workers using multivariate analysis techniques. In 1975, a relatively simple computer program (3) was used to differentiate among coldpressed orange peel oils previously separated into three classes by sensory evaluation. Use of quantitative data for 63 oil constituents and ratios of combinations of two and three constituents totalling 120 values for each oil sample, afforded correct

classification for all oils analyzed. Such time-intensive calculations were not suitable for routine quality control analysis.

A later study on orange essence oils made use of multivariate statistical and pattern recognition techniques (chemometrics) available in the ARTHUR chemometrics program to classify 45 samples (2). In that study, 24 Valencia orange essence oils and 21 navel orange essence oils were separated correctly into two distinct classes. Up to 160 data values from a gas chromatogram of each oil were analyzed by the ARTHUR program, and four constituents were determined to be most important in classifying samples. Two of the four constituents were identified as *trans*-2-hexenal and terpinene-4-ol, but the remaining two were only partially identified as a polyunsaturated hydrocarbon and a dicyclic sesquiterpenoid.

Attempts to classify orange juice samples by statistical treatment of analytically determined values have been carried out over the past 20 year period. In a major orange "juice definition program" carried out by research scientists at the Florida Department of Citrus over a period of several years (4-6), most of the statistically significant parameters measured were related to the nonvolatile constituents by multiple regression. These included limonin, metal ion concentration and acidity, as well as total sugars, pectins, protein and glycosides (4,5). However, in a later study using the same database (6), total oil and oxygenated terpenes were two of the more important parameters, and total aldehyde measurement was a significant analytical parameter for correlating juice flavor over two seasons. These three parameters were all related to volatile constituents present even though no single volatile constituent was monitored.

Rouseff and Nagy (7) studied samples from the juice definition program described above using 34 measurements to analyze 105 samples with the ARTHUR program for multivariate analysis. The best measurements for classifying the juices into the proper flavor categories were limonin, formol number (total amino nitrogen), potassium, total sugars and total oil content. As found above in the earlier studies, most of the important factors for classification were due to nonvolatile constituents. Only the total oil reflected the contribution by volatile flavor constituents. Three eigenvectors determined using these five factors accounted for 97% of the variance. Quantities of individual volatile constituents were not measured in those studies, and thus none were available for multivariate analysis. Pino (8) used multiple linear regression involving seven orange volatiles from juice quantified by gas chromatography to select five of the constituents (limonene, linalool, α-terpineol, myrcene and 2-hexanol) whose variabilities were most significant in explaining sensory differences. Equations involving the first three constituents plus myrcene explained 96%, while those involving the first three constituents plus 2-hexanol explained 94% of the observed aroma differences.

Several multivariate analysis procedures were used to compare classifications of 16 samples of natural orange aroma by sensory evaluation and by quantitative gas chromatographic analysis of 30 volatile constituents (9).

Natural orange aroma contains the water-soluble volatile flavor constituents of orange juice, and is commercially used to flavor concentrated orange juice products (10). A nonlinear mapping program using gas chromatographic data was able to classify all samples into nine categories. Aroma data obtained with a trained sensory panel was less discriminatory; off-aroma samples were classified into distinct categories, but the better quality samples could not be positively placed in distinct categories in all cases. Analysis by SIMCA (Statistical Isolinear Multicategory Analysis) was more effective than was KNN (K-nearest neighbor) in classification using sensory data (9).

Related Studies for Determining Place of Origin and for Detecting Adulteration.

Country of origin was determined for several samples of frozen concentrated orange juice (FCOJ) from either Florida or Brazil (11). Based on earlier results showing concentrations of 5 elements as significant in distinguishing between geographical locations (12), a relatively simple pattern recognition program involving a "decision vector" clearly discriminated between concentrated juices from Florida and Brazil. Nikdel and co-workers (13) used the ARTHUR pattern recognition package to determine place of origin for orange juices based on quantities of 17 elements present in each juice. They were able to distinguish juices from the following places of origin: Florida vs. Brazil, Florida vs. Belize; Florida vs. Mexico, and Florida vs. California vs. Arizona.

The same group (14) used a different pattern recognition program (EinSight) for detection of orange juices adulterated with grapefruit juice. Principal Component and Complete Link Farthest Neighbor Cluster analysis of autoscaled data using EinSight software permitted differentiation of orange and grapefruit juices and mixtures containing 50% or more grapefruit juice. Page (15) described a protocol for determining adulteration in, or country of origin for orange juice samples. He was able to discriminate with a high degree of success among California, Florida and adulterated juices, as well as authentic, adulterated and pulpwash-containing orange juice.

HEADSPACE GAS CHROMATOGRAPHY WITH MULTIVARIATE ANALYSIS

In studies at our laboratory, a static headspace gas chromatographic (HS-GC) system was used to collect quantitative data on from 19 to 25 volatile orange juice constituents. Multivariate analysis with the EinSight program was used to perform Principal Component analysis and Cluster analysis on autoscaled data (16).

Initial Study Involving 19 Volatile Constituents Juice Samples. The eighteen commercial juice samples listed in Table 1 were used in this study (17). Four samples of fresh, unpasteurized juice were studied (F1-F4 in Table 1) including three "fresh-squeezed" commercial samples with a 17-day expiration date

from time of juice extraction (18) and one fresh Valencia orange juice sample taken directly from a local commercial juice extraction line. Five samples were pure premium pasteurized juices not from concentrate (P1-P5). Seven samples from orange concentrate included three (C1-C3) from frozen concentrated orange juice (FCOJ). One asceptically packaged concentrate (C4) and three single-strength juices from concentrate, one each packaged in glass, fiberboard carton, and tin can (R1-R3).

Two aseptically packed samples were single-strength juice from concentrate packaged in flexible multilayered 250-mL cartons (samples A1 and A2). All

Table 1. Eighteen Juice Samples Evaluated in Initial Study

Sample[a]	Type of juice[b]	Packaging
F1	fresh-squeezed[c]	plastic bottle
F2	fresh-squeezed	plastic bottle
F3	fresh-squeezed	plastic bottle
F4	unpasteurized[d]	plastic bottle
P1	pasteurized NFC[e]	flexible gable-top
P2	pasteurized NFC	flexible gable-top
P3	pasteurized NFC	flexible gable-top
P4	pasteurized NFC	flexible gable-top
P5	pasteurized NFC	flexible gable-top
C1	frozen concentrate[e]	paperboard with metal ends
C2	frozen concentrate	paperboard with metal ends
C3	frozen concentrate	paperboard
C4	aseptic pack concentrate[f,g]	laminated multilayer
R1	single strength from concentrate	glass
R2	single strength from concentrate	flexible gable-top
R3	canned	tin can
A1	aseptic pack[g]	laminated multilayer
A2	aseptic pack[g]	laminated multilayer

[a]Indicates the code used for each sample in Figs. 1, 3 and 4.
[b]Commercially packaged for retail market unless otherwise noted.
[c]Commercially packaged unpasteurized juices purchased on April 27 (F1), April 17 (F2) and April 20 (F3).
[d]Fresh unpasteurized Valencia juice commercially extracted on April 3, 1992.
[e]NFC, not from concentrate.
[f]Reconstituted to 11.8° Brix juice prior to analysis.
[g]Packaged aseptically in 250-mL rectangular flexible carton.

commercially packaged samples were purchased at local markets except for one aseptically packaged single-strength juice which was obtained directly from a processing line, and the aseptically packaged concentrate which was provided by a processor. All samples were stored at -18°C until analyzed.

Headspace GC Analysis of Juice. A 2-mL sample of juice in a 10-mL vial sealed with a crimptop cap with TFE/silicone septum seal was equilibrated for 15 minutes at 80°C in a Model HS-6 headspace sampler attached to a Perkin-Elmer Model 8500 GC with an FID detector. A 0.53mm x 30m polar Durowax column with 1.0μm film thickness (J&W Scientific, Folsom, CA) was used with helium carrier gas at a head pressure of 41 kPa (81 cm/s linear gas velocity). Injection conditions for the headspace sampler were 0.5 min. vial pressurization time followed by 0.02 min injection time. The GC was temperature programmed at 40°C isothermally for 6 minutes, then 6°C/minute to 180°C. The FID amplifier range was set for high sensitivity and the detector temperature was 250°C. All determinations were carried out in triplicate or quadruplicate. Individual constituents were identified by comparison of retention times with those for standards and by enrichment of juice with authentic samples. Concentrations for 19 constituents were calculated with regression equations, determined by injecting five different concentrations of each constituent added to a juice base to obtain a peak height calibration curve as described previously (19). Concentrations for each constituent were chosen to span the range of quantities reported earlier in orange juice (19). The juice base was prepared by reconstitution to 11.8°Brix from concentrated juice (pumpout) from an evaporator that contained no added flavor fractions.

Multivariate Analysis. The EinSight Version 3.0 data analysis and pattern recognition program from Infometrix, Inc., Seattle, WA, was used in this study. Individual values in ppm for the 19 constituents quantified in each of the 18 juice samples were entered into a spreadsheet (Quattro Pro Version 3.0, Borland Int'l., Inc., Scotts Valley, CA) prior to multivariate analysis.

Results. Eighteen commercially produced fresh and processed orange juice samples were analyzed by headspace GC and the quantities of 19 constituents determined by this method were analyzed by a computer multivariate pattern recognition program. This program calculated variation among all of the 19 volatile compounds quantified to differentiate the following four categories of orange juice: unpasteurized, pasteurized not from concentrate, frozen concentrate, or juices reconstituted from concentrate.

Principal component analysis of the 19 compounds quantified by headspace GC afforded the eigenvector report in Table 2 using autoscaled data for the first five eigenvectors (principal components). Autoscaling of data removes the differences caused by large variation in reporting units, or in cases such as the current one, differences in magnitude for different components Autoscaling allows all variables to be compared on the same normalized standard distribution (20). The eigenvector report in Table 2 shows that principal component 1 (PC-1) contains 25% of the total variation among juice samples represented by the 19

components quantified. PC-1 is the linear expression which contains the maxium amount of variation among the 19 volatile constituents (1).

Table 2. Eigenvector Report from Autoscaled Data

Principal Component	% Variation	% Cumulative Variation
1	25.0	25.0
2	19.8	44.8
3	13.2	58.0
4	11.8	69.8
5	7.4	77.2

Two Dimensional Plots. The second principal component (PC-2) is chosen orthogonal to first principal component, and contains the greatest remaining variation among the 19 components that is unrelated to PC-1. From Table 2, PC-2 contains 19.8% of the total variation, and PC-1 and PC-2 together contain 44.8% of total variation. They (PC-1 vs PC-2) can be plotted in two-dimensional space as shown in Fig. 1. Straight-line boundaries shown on this figure separate the juice types into four categories, where F1-F4 indicate the four fresh, unpasteurized juices, P1-P5 indicate the five pasteurized juices not from concentrate, C1-C4 and R1-R3 indicate the juices from concentrate, and A1-A2 indicate the aseptically packed juices, also from concentrate.

The "loading" for each compound is the relative contribution of that compound to a specific principal component. Fig. 2 is a plot of PC-1 vs PC-2 showing the loadings of all 19 compounds for these two principal components. Methyl butyrate, ethyl butyrate, and ethyl acetate rate high on the first principal component and are very close together, indicating they provide similar information (1). This is in keeping with the belief that these volatile esters contribute to the desirable fresh "fruity" note in good quality orange juice (21). Similarly, *cis*-3-hexenol and linalool rate high on the second principal component, and are very close together on the plot. They are both known to contribute fresh top notes to fruity and flowery aromas (22, 23).

Many of the compounds ranked highest in loadings 1 and 2 are known to be important to fresh flavor in good quality juice (19, 21, 24). In addition to the three esters discussed above, these include acetaldehyde and ethanol. Relatively lower amounts of these compounds in more highly processed juice such as those reconstituted from concentrate helps explain observed differences in juice categories.

A three dimensional plot of principal components 1-3 was displayed with EinSight, also (not shown). Although principal components 1-3 represent 58% of

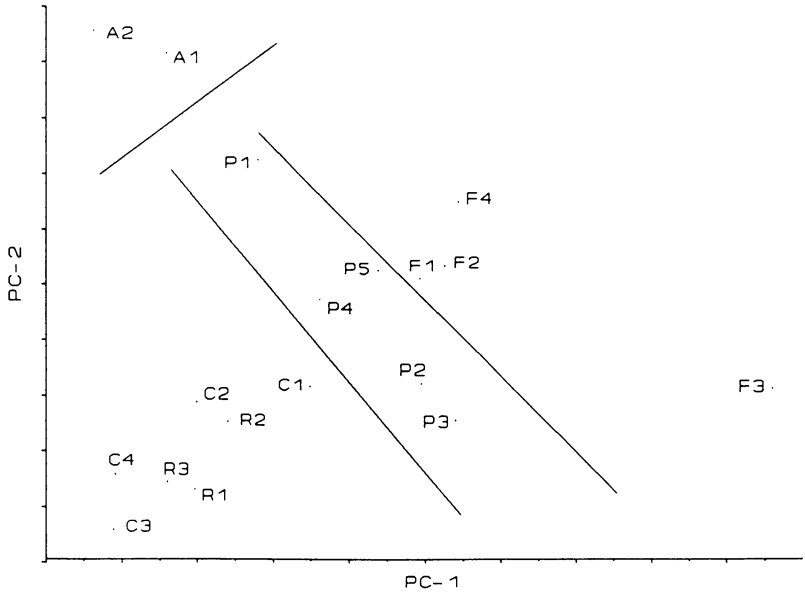

Figure 1. Plot of PC-1 vs PC-2 for 19 compounds monitored in commercial orange juice.

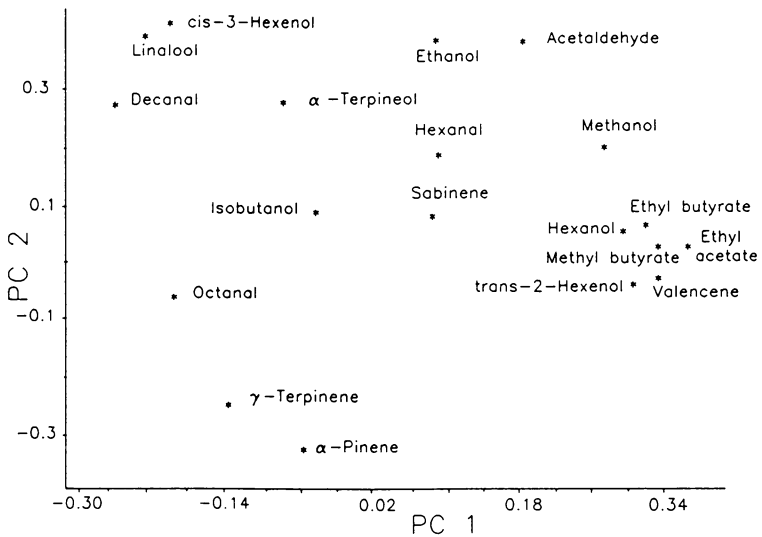

Figure 2. Two dimensional plot of loadings for PC-1 vs PC-2 for 19 compounds showing correlations between variables and principal components (20).

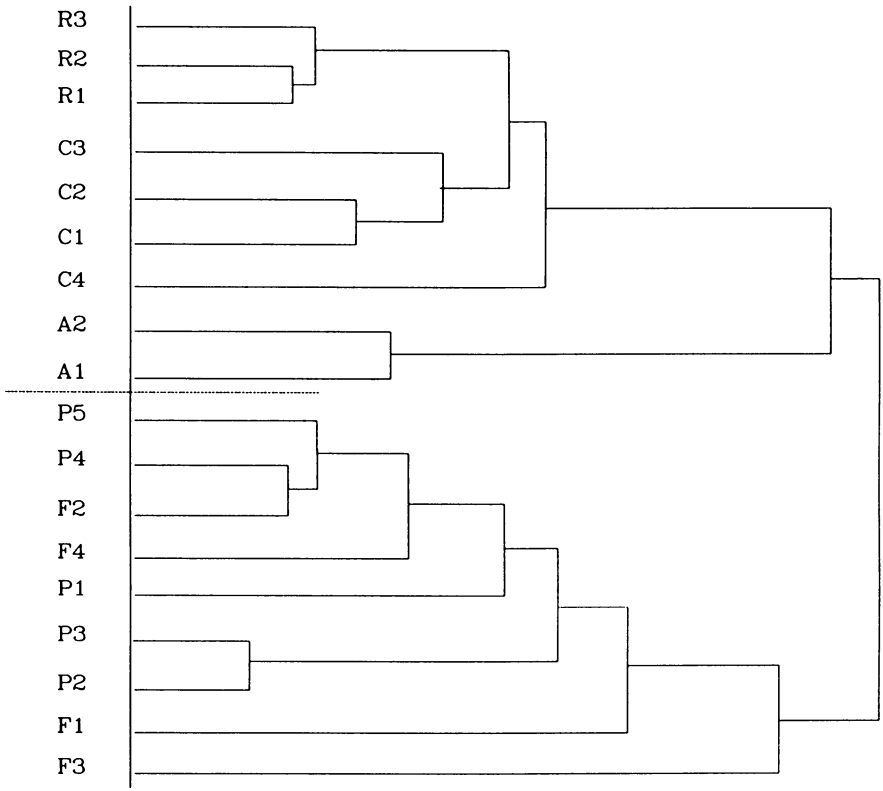

Figure 3. Dendrogram showing Incremental Cluster Analysis for 19 volatile compounds in 18 juice samples showing separation into two classes (dotted horizontal line). Table 1 identifies juice samples classified.

the total variance (Table 2), the separation of classes was no better than with PC-1 and PC-2 in Fig. 1. The effect of the third dimension (z-axis for PC-3) was not readily apparent, either on the computer monitor or in the two-dimensional printout. Three dimensional projections such as those published by Lin et. al., (9) using the ARTHUR program are not available with EinSight.

Another method for categorizing samples with many pattern recognition programs, including EinSight, involves cluster analysis to produce a dendrogram (Fig. 3). Of the eight different types of dendrograms that can be generated, only "Incremental Sum of Squares" separated the juices completely into two categories at a similarity value of 0.07. The fresh-squeezed and the pasteurized, not from concentrate juices together were separated from the reconstituted juices from concentrate and frozen concentrated orange juices.

Later Study Involving 25 Volatile Constituents

The basic procedure used in the above study was modified by quantification of six additional orange juice constituents for a total of 25 constituents quantified in each of 27 commercial juice samples listed in Table 3. The six additional constituents quantified were myrcene, limonene, ethyl hexanoate, ethyl 3-hydroxyhexanoate, octanol and terpinene-4-ol. The two terpene hydrocarbons, myrcene and limonene, were found to be two of the most important constituents in principal component analysis to classify these 27 juice samples.

Modification of Standardization Procedure. In order to obtain accurate values for the six hydorcarbons quantified, the procedure for preparation of the standard mixtures used in determination of the standard curves was modified from that described above. A standard solution of all 25 compounds to be quantified (listed in Table 4) was prepared in ethanol. Aliquot portions of this standard solution were added to single strength (11.8° Brix) orange juice base prepared from orange juice concentrate (evaperator pumpout), which contained no other added flavor constituents. The standard mixture, thus prepared, was allowed to stand for 3 hours at room temperature and then kept for 18 hours at 5°C prior to headspace GC analysis. In preliminary tests, this period of time was necessary to permit the added terpene hydrocarbons to equilibrate between pulp and serum of the juice. The quantities of the hydrocarbons present in the headspace gases decreased steadily and significantly in the first 3 hours and then reached a fairly steady state. However, they reached a final equilibration only after standing overnight in a refrigerator. None of the 19 oxygenated compounds in the standard mixture were affected by this equilibration period. Previous reports had indicated the presence of such an equilibration between pulp and juice serum for peel oil (25), and for hydrocarbon components in particular (26). Duplicate GC runs at four different concentrations selected to cover the range of values found in the 27 juice samples were employed in determining standard curves.

Results. In terms of classification and separation of the types of commercial juices, the results were similar to those reported above for an earlier

Table 3. Twenty-seven Juice Samples Evaluated in Later Study

Samples[a]	Type of juice
F1-F3	fresh squeezed[b]
F4	unpasteurized[c,d]
F5	unpasteurized[e]
P1-P6	pasteurized NFC[f]
C1-C5	frozen concentrate[g]
C6-C7	frozen concentrate[d,g]
R1-R5	single strength from concentrate[d]
A1-A4	single strength from concentrate[d,h]

[a]Indicates code used for each sample in Figs. 5 and 6.
[b]Unpasteurized juice commercially packaged in plastic bottle.
[c]Commercially extracted unpasteurized Valencia juice stored in plastic bottle.
[d]Obtained directly from processor.
[e]Unpasteurized hand extracted Pera juice stored in glass.
[f]NFC, not from concentrate and commercially packaged in flexible carton.
[g]Reconstituted to 11.8° Brix juice prior to analysis.
[h]Aseptically packaged in single serve container.

study. However, some important differences in the two studies were found, especially regarding some of the newly quantified constituents. Principal component analysis afforded the plot for PC-l vs PC-2 shown in Fig. 4. As found in an earlier study, three broad juice classifications were observed. They are fresh-squeezed unpasteurized (F values in Fig. 4), pasterized not from concentrate (P) and juices prepared from concentrate, whether sold as frozen concentrated juice (C) or reconstituted from concentrate (R and A). PC-l and PC-2 together contained 56% of the total variation represented by the 25 constituents monitored. If PC-3 was considered, an additional 10% of the variation was included.

The three dimensional plot of PC-l vs PC-2 vs PC-3 is shown in Fig. 5. The separation observed is similar to that observed for the two dimensional plot. As in the previous example the third PC does not describe enough distinct variation to make a noticeable difference in this plot.

Loadings. In addition to a loadings plot such as described above for Fig. 2, a listing of all constituents used in the calculations and their relative loadings is obtained. Loadings for the first three principal components are shown in Table 5. Four of the six additional components quantified in this later study were among the most important compounds in at least one of the three most significant principal components. Thus, myrcene and octanol are two of the four constituents with highest loadings values in PC-1. Limonene in PC-2 and ethyl 3-hydroxyhexanoate in PC-3 each had the second highest loadings values of any

Table 4. Amounts of Volatile Compounds (parts per million) in Three Commercial Orange Juice Types

	juice type					
	fresh[a]		pasteurized[b]		from concentrate[c]	
aldehydes						
acetaldehyde	11.1	8.6-13.8	8.6	5.6-11.3	2.4	tr.-8.5
hexanal	.28	0.14-0.61	.22	0.09-0.28	.06	tr.-0.17
octanal	.80	0.02-1.4	.78	0.29-2.1	.50	0.06-0.93
decanal	.64	0.02-1.2	.53	.0.14-1.5	.60	0.02-1.4
esters						
ethyl acetate	.33	0.18-0.64	.19	0.11-0.30	.12	tr.-0.43
methyl butyrate	.001	0.006-.03	.007	tr.-0.009	.005	tr.-0.43
ethyl butyrate	.79	0.60-1.01	.49	0.19-0.75	.25	tr.-0.015
ethyl hexanoate	.11	0.07-0.14	.054	0.01-0.08	.056	tr.-0.76
ethyl-3-hydroxy-hexanoate	1.4	1.1-1.8	2.5	0.64-9.4	2.1	tr.-0.1
alcohols						
methanol	61	5-108	30	4-40	13	tr.-30
ethanol	730	520-1150	610	280-860	240	tr.-580
2-methyl-1-propanol	.038	0.01-0.09	.011	tr.-0.024	.040	0.008-.14
hexanol	.21	0.05-0.41	.041	tr.-0.11	.009	tr.-0.026
cis-3-hexanol	.56	0.35-0.80	.52	0.31-1.0	.44	0.13-0.65
trans-3-hexanol	.001	tr.-0.002	tr	tr	tr.	tr.
linaloonl	1.5	0.15-2.25	1.5	0.75-3.3	1.2	0.09-2.3
octanol	.43	0.001-.65	.2	0.016-0.4	.11	0.007-.24
terpinene-4-ol	.68	0.09-1.6	.48	0.07-0.92	.38	0.09-0.73
α-terpineol	.75	0.31-1.75	.50	tr.-1.0	.25	0.02-0.9
hydrocarbons						
limonene	159	19-301	152	57-317	132	tr.-215
myrcene	4.2	0.85-5.7	3.2	1.1-7.0	2.8	tr.-4.3
α-pinene	.94	0.36-1.4	.74	0.42-1.2	.63	0.16-101
sabinene	.35	0.06-.065	.02	tr.-0.09	0.08	tr.-0.33
γ-terpinene	.024	tr.-0.056	.018	.0003-.034	.017	.0004-.041
valencene	11.2	7.8-16.4	7.9	6.1-8.5	1.83	0.65-3.0

[a]Five unpasteurized juice samples: four commercial "fresh squeezed" samples and one commercially extracted and finished Valencia juice.
[b]Six pure premium pastueurized juice samples, not from concentrate.
[c]Sixteen samples, seven from frozen concentrated orange juice and nine single strength juices from concentrate.

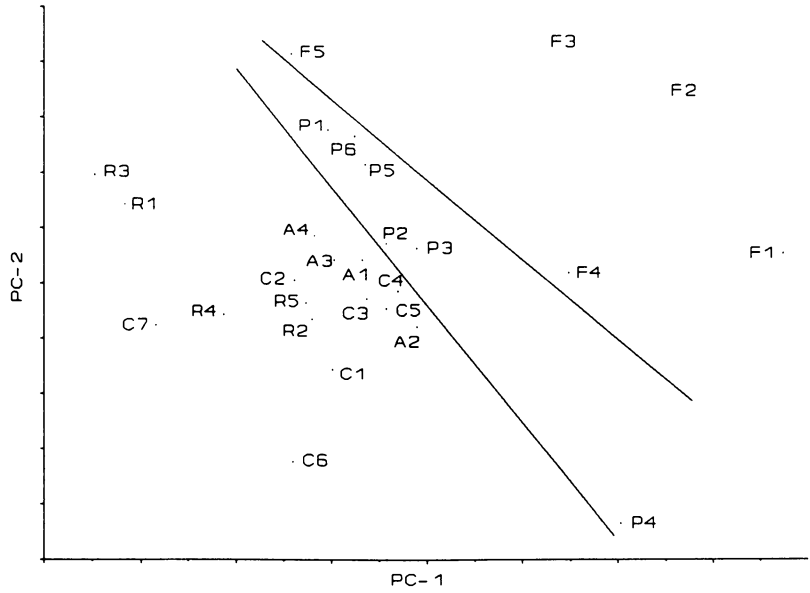

Figure 4. Two dimensional plot for principal component analysis of 25 compounds monitored in 27 commercial orange juices. Table 3 identifies juice samples classified.

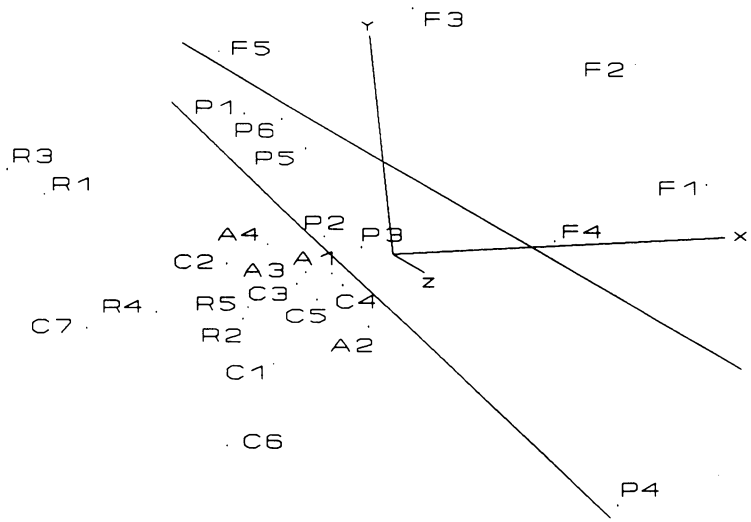

Figure 5. Three dimensional plot for 27 juice samples in Figure 4.

Table 5. Loadings for First Three Principal Components

PC-1		PC-2		PC-3	
Constituent	Loading	Constituent	Loading	Constituent	Loading
Methanol	0.277	Decanal	-0.360	Sabinene	-.0432
Mycene	0.246	Limonene	0.311	Ethyl 3-hydroxy-hexanoate	0.395
α-Pinene	0.261	*cis*-3-Hexanol	-0.278	Methyl butyrate	0.394
Octanol	0.251	Valencene	0.263	2-Methyl-propanol	-.340
Octanal	0.241	Hexanol	0.258	α-Terpineol	-.321
cis-3-Hexenol	0.240	Octanal	-.243	Ethyl acetate	0.286
Hexanal	0.237	Myrene	-0.227	Linalool	0.244
Acetaldehyde	0.235	Ethyl 3-hydroxy-hexanate	-0.214	Ethyl butyrate	0.175
Ethanol	0.234	Acetaldehyde	0.213	Ethanol	0.166
Limonene	0.224	*trans*-2-Hexenol	0.203	α-Pinene	-0.136
Linalool	0.210	Linalool	-0.198	Terpinene-4-ol	-0.132
γ-Terpinene	0.207	Ethyl acetate	0.192	Ethyl hexanoate	0.129
Hexanol	0.204	Methyl butyrate	0.188	Hexanol	-0.113
Terpinene-4-ol	0.202	2-Methyl-1-propanol	-0.171	*trans*-2-Hexenol	-0.099
Ethyl Acetate	0.198	α-Pinene	-0.167	Methanol	-0.049
α-Terpineol	0.194	γ-Terpinene	-0.150	Myrcene	-0.045
Ethyl hexanoate	0.186	Ethyl hexanoate	0.149	Decanal	-0.035
Valencene	0.168	Ethanol	0.148	Acetaldehyde	0.034
Sabinene	0.167	Octanol	0.135	Limonene	0.033
Methyl Butyrate	0.148	Hexanal	0.129	Hexanal	-0.024
Decanal	0.144	Methanol	0.128	Octanol	0.022
Ethyl 3-hydroxy-hexanoate	0.082	α-Terpineol	0.110	Valencene	-0.019
2-Methyl-1-propanol	0.079	Sabinene	0.101	Octanal	-0.006
trans-2-Hexenol	0.051	Terpinene-4-ol	0.084	γ-Terpinene	0.005
Ethyl Butyrate	0.050	Ethyl butyrate	0.048	*cis*-3-Hexenol	-0.002

constituent for those principal components. However, inclusion of these additional components with high loadings values did not change the basic classifications from those found above in the initial study.

Cluster Analysis. Dendrograms produced by Hierarchical Cluster Analysis using seven different clustering techniques were not able to completely separate the 27 juice samples even into two broad categories (clusters). The best separation into two clusters was achieved by Single Link and by Complete Link clustering techniques. The classes were unpasteruized and pasteurized not from concentrate as one broad class and juices from concentrate as a second broad class. In both clustering techniques, two of the 27 juice samples were misclassified. Thus, principal component analysis was a more effective technique for classifying the juice samples.

Conclusions

Principal component analysis using 19-25 constituents of two sets of commercial orange juice samples has separated the juices into three broad categories, which were fresh-squeezed unpasteurized juice, pasteurized not from concentrate juice and juice from concentrate. These three categories of juice are considered by consumers to be different in flavor quality, and are priced accordingly. Fresh-squeezed juice is priced highest, with pasteurized juice not from concentrate intermediate in price and juices from concentrate generally the lowest priced, corresponding to their perceived flavor differences.

Acknowledgements

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Chapter 5

Characterization of Carambola and Yellow Passion Fruit Essences Produced by a Thermally Accelerated Short-Time Evaporation Citrus Evaporator

Steven Nagy[1], Sandy Barros[1], and Chin Shu Chen[2]

[1]Scientific Research Department, Florida Department of Citrus, Citrus Research and Education Center, Lake Alfred, FL 33850
[2]University of Florida, Citrus Research and Education Center, Lake Alfred, FL 33850

Juices from carambola and yellow passion fruit were concentrated to 62^0 Brix and 44-46^0 Brix, respectively, using a 3-effect, 4-stage pilot-plant TASTE citrus evaporator. Juice essences were recovered during the concentrating process by use of an essence recovery system. Carambola essence possessed an unripe apple-like or apricot-like aroma. Examination by gas chromatography-mass spectrometry revealed ethyl acetate, trans-2-hexenal, cis-3-hexenol, trans-2-hexenol, n-hexanol and several minor alcohols, esters, and terpenes. Yellow passion fruit essence revealed 41 identifiable components. Esters were present in abundance as well as high levels of alcohols. Aliphatic C_1-C_8 alcohols (linear, secondary, unsaturated, iso- and anteiso-branched) and terpinyl alcohols (linalool, terpinen-4-ol, α-terpineol, nerol, geraniol) were noted. The potential use of these flavor fractions in tropical beverage formulations appears promising.

The tropical fruit industry in Florida is expanding and producing a variety of exotic fruits (Table I) valued at about $70-80 million (1). Currently, the market for these fruits is mainly limited to fresh consumption. Any fruit that does not meet grade standards for the fresh fruit market is subsequently discarded. Therefore, a need exists to salvage these packinghouse eliminations and develop processing technologies that will yield value-added juice products and byproducts. Citrus processing facilities are potentially suitable, with some modifications, for the processing of tropical fruits.

This paper describes a pilot plant feasibility study to produce juice concentrate and essence from two tropical fruits, namely, carambola and yellow passion fruit. The characterizations of these produced tropical fruit essences are the major focus of this paper.

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Table I. Commercial Tropical Fruit Crops in Florida

Common Name	Scientific Name	Average
Atemoya	Annona cherimola x A. squamosa	75
Avocado	Persea americana	6,000
Banana (eating)	Musa spp.	400
Barbados cherry	Malpighia glabra	37
Black sapote	Diospyros ebenaster	1
Canistel	Pouteria campechiana	1
Carambola	Averrhoa carambola	400
Guava	Psidium guajava	100
Jaboticaba	Myrciaria cauliflora	1
Jackfruit	Artocarpus heterophyllus	5
Key lime	Citrus aurantifolia	15
Kumquat	Fortunella spp.	25
Tahiti lime	Citrus latifolia	2,500
Longan	Nephelium longana	100
Lychee	Litchi chinensis	150
Macadamia	Macadamia spp.	1
Mamey sapote	Pouteria sapota	200
Mango	Mangifera indica	1,000
Papaya	Carica papaya	450
Passionfruit	Passiflora edulis & f. flavicarpa	10
Pummelo	Citrus grandis	40
Sapodilla	Manilkara zapota	30
Sugar apple	Annona squamosa	30
Minneola tangelo	Citrus reticulata x C. paradisi	5
Wax jambu	Syzygium smarangense	1
White sapote	Casimiroa edulis	2

Source: (Crane, J.H., University of Florida, personal communication, 1993).

CARAMBOLA

Description

Carambola (*Averrhoa carambola* L.), also known as star fruit and other names specific to different geographical locations, is a member of the family Oxalidaceae. Major production areas in the world are the tropical and subtropical regions in East Asia, including Indonesia, Malaysia, Taiwan, Guangdong and Fijian Provinces in southern China. It is also grown in certain regions in West Tropical Africa, Australia, South and Central Americas, and the southern part of Florida.

The fruit is ovoid to ellipsoid, 6-15 cm in length, with 5 (rarely 4 or 6) prominent longitudinal ribs, and star-shaped in cross section (Figure 1). Unripe fruits are green and firm, turning yellow, orange or amber, and soft during ripening. The flavor of carambola is variable, and ranges from sour with little sugar to sweet with little acid (2). Wagner et al. (3) reported ascorbic and oxalic acid contents, acidity, Brix, and taste panel evaluation for carambolas that were mostly of the yellow varieties.

Processing Procedures

Juice was extracted from ripe carambolas (cv. Arkin) at about 4°C using a screw-type finisher (FMC Model 35 finisher with reinforced .027 in. finisher screen openings). Juice quality indicators were developed for carambola and yellow passion fruit (Table II). Quality indicators for Valencia orange juice were included for comparative purposes. Single-strength carambola juice was concentrated to 62° Brix using a 3-effect, 4-stage TASTE (Thermally Accelerated Short Time Evaporation) citrus evaporator. The evaporator was designed to combine the pasteurization needed to deactivate microorganisms and enzymes, and to remove water in a continuous manner by evaporation. Evaporator water removal capacity was 500 lbs per hr. The TASTE evaporator was designed to pasteurize juice at about 100°C for 10 sec. Additionally, this evaporator was equipped with an essence recovery system that recovered the carambola essence (4).

Table II. Analytical Quality Indicators for 'Arkin' Carambola, Yellow Passion Fruit and Valencia Orange

Indicator	Carambola[a]	Yellow Passion Fruit[b]	Valencia Orange[c]
°Brix (B)	7.80	15.50	13.10
% Acid (A)	0.34	3.94	1.03
B/A Ratio	22.85	3.93	12.90
% Pulp	9.00	20.00	4.00
pH		2.90	3.70
% Light Trans.	43.00	4.00	8.00
Brookfield Viscosity (cps)		20.00	1.70
Total Glycosides (mg/100ml)	0.045		115.00
% Oil	<0.0004	0.0008	0.015
Color Number	32.10		37.60

[a]From Nagy et al. (10).
[b]From Chen et al. (9).
[c]From Barros (16).

Essence Analyses

Aqueous essence samples isolated from the essence recovery unit were chromatographed on a Hewlett-Packard Model 5890 gas chromatograph fitted with a 0.32 mm i.d. x 30 m bonded-phase, nonpolar RTX-5 fused silica capillary column (1 micron film; Restek Corp., Bellefonte, PA). The injection port and flame ionization detector were set at 240°C and 250°C, respectively. Hydrogen carrier gas flow rate was about 41 cm/sec and the injection split ratio was 50:1. Samples were injected at a temp. of 32°C and held 3 min; then temp. programmed from 32°C to 230°C at 7.5°c/min. Essence components were identified by comparison to retention times of authentic compounds and by mass spectral identification utilizing the Kratos MS 25 magnetic sector, double focusing mass spectrometer.

Carambola Essence Evaluation

Essences were characterized from fruit of two different harvests, namely, from fruit harvested and processed in March 1990 and from fruit of the October 1990 harvest. The essence recovery unit of a TASTE citrus evaporator separates essences into two phases: an oil phase (essence oil) and an aqueous essence phase (industrially termed 'aroma'). We could distinguish no distinct oil phase; however, an aqueous essence phase was readily apparent. Table III lists the aqueous essence constituents from the two harvests. Twenty-three compounds were identified by GC/MS. The aroma of carambola aqueous essence recovered from the TASTE essence recovery unit possessed a sweet, fruity, unripe apple-like note. This aroma note is apparently the result of a combination of acetates (methyl acetate and ethyl acetate) that impart pleasant, fruity-sweet odors and compounds (hexanal, trans-2-hexenal, cis-3-hexen-1-ol, trans-2-hexen-1-ol and 1-hexanol) that impart fresh green, grassy unripe notes (Table IV).

The odor description of acetaldehyde is that of a pungent, penetrating ethereal note. Its aroma role in carambola essence is not defined. In natural orange aroma, acetaldehyde is quite important as a quality impact factor, however (5). The dominant alcohols, methanol and ethanol, impart a slight, sweet ethereal note. Since the odor of ethanol is perceptible in aqueous solutions down to about 12%, it may not be important at the low concentration levels found in these two essences, namely, 2.4% (harvest A) and 0.1% (harvest B). Although methanol and ethanol do not appear to impact directly on the aroma note of carambola essence, they may in fact increase overall aroma perception by enhancing (lifting) other aroma components as occurs in perfume formulations.

Few studies have been conducted on the identity and quantitative estimation of volatile flavor components in carambola. Wilson et al. (7) noted that the aroma of the essence extracted from carambola puree by methylene chloride possessed a strong floral fruity aroma (grape-like, fruity and aromatic). Those workers identified 43 compounds comprised of 13 alcohols,

4 aldehydes, 6 ketones, 13 esters, 3 hydrocarbons and 4 compounds classified as miscellaneous. Methyl anthranilate, the major volatile isolated by Wilson and colleagues (7), imparted a characteristic grape-like note.

Table III. Carambola Essence Constituents Recovered by Essence Unit of the TASTE Citrus Evaporator

Component	RT (min)	Rel. Area % A[a]	Rel. Area % B[b]
acetaldehyde[c]	1.38	0.60	15.66
methanol[c]	1.38	3.64	2.14
ethanol	1.70	86.28	54.61
acetone	1.97	0.26	0.06
2-propanol	2.00	1.23	0.40
methyl acetate	2.37	0.02	0.37
1-propanol	2.78	0.06	0.10
ethyl acetate	3.87	0.70	2.51
2-methyl-1-propanol	4.20	0.06	0.23
1-butanol	5.16	0.02	tr
1-penten-3-ol	5.62	0.05	0.35
3-methyl-1-butanol	7.15	0.01	0.11
1-pentanol	8.21	0.02	tr
hexanal	8.95	0.02	0.37
ethyl butyrate	9.00	0.01	0.08
trans-2-hexenol	10.52	4.79	13.80
cis-3-hexen-1-ol	10.61	0.39	0.58
trans-2-hexen-1-ol	10.89	1.09	1.74
1-hexanol	10.94	0.30	1.06
1-octanol	16.11	0.03	0.18
linalool	16.88	0.03	0.18
α-terpineol	19.12	0.02	0.14
carvone	20.30	0.02	0.08

[a]Sample processed March 12, 1990
[b]Sample processed October 30, 1990
[c]Acetaldehyde and methanol were resolved on 6′ x 1/4″ glass column packed with 5% Carbowax 20 M on 80/120 mesh Carbopack BAW.
From Nagy et al. (10)

SOURCE: Permission has been verbally granted by Dr. Norman Childers, editor of the Florida State Horticultural Society.

Table IV. Odor Descriptions for Selected Constituents in Carambola Aqueous Essence

Constituent	Odor Description[a]
acetaldehyde	pungent, penetrating ethereal-nauseating odor
methanol	mild sweet etheral-like
ethanol	alcoholic, sweet etheral
2-propanol	alcoholic, somewhat unpleasant odor
methyl acetate	fruit-sweet, pleasant, reminiscent of pineapple
hexanal	fatty green grassy, unripe fruit
cis-3-hexen-1-ol	intense green, grassy odor
trans-2-hexen-1-ol	powerful, fruity, green, leafy odor
1-hexanol	mild, sweet, green

[a]From Furia and Bellanca (6)

Recent flavor research on star fruit (carambola) by Winterhalter et al. (8) indicates that carotenoid derived compounds are important contributors to overall flavor impression. Norisoprenoid volatiles in star fruit were derived from glycosidically bound precursors. The intensely odorous c_{13} ketone, β-damascenone, was identified. Wilson and coworkers (7) were able to identify only one norisoprenoid volatile in star fruit, namely,β -ionone.

YELLOW PASSION FRUIT

Description

Yellow passion fruit (*Passiflora edulis* Sims *F.flavicarpa* Degener) is a member of the family Passifloraceae. The fruit is called yellow lilikoi in Hawaii; golden passion fruit in Australia; parcha amarilla in Venezuela. The chief producing countries in recent years are Australia, Hawaii, South Africa, Kenya, Columbia, Venezuela, The Philippines, Fiji and Brazil.

The fruit (Figure 2) is round or ovoid, 4-7.5 cm wide, and has a tough rind that is light-yellow or pumpkin-color. The pulp is highly aromatic, acidic, juicy, and contains large brown seeds.

Processing Procedures

Yellow passion fruit juice produced in Brazil was centrifuged, pasteurized, chilled and packed in 55-gal drums. Twelve drums of single-strength juice were frozen

Figure 1. Carambola Fruits
SOURCE: Reproduced with permission from AgScience, Inc.

Figure 2. Yellow Passion Fruit-Vine, Flowers and Fruit
SOURCE: Reproduced with permission from AgScience, Inc.

and shipped to Florida. The drums arriving in Florida were thawed at room temperature and the juice transferred into a holding tank for processing. The thawed juice was fed to a 3-effect 4-stage pilot TASTE citrus evaporator. The initial juice solids was 15.5^0 Brix (% soluble solids by weight) and after concentration of the whole juice, the evaporator concentrate had juice solids in the range of $44\text{-}46^0$ Brix. Both an oil phase and an aqueous phase essence were recovered from whole juice by the essence recovery system (9). However, only the aqueous essence phase was investigated in this study.

Essence Analysis

Aqueous essence from yellow passion fruit was characterized by gas chromatography and by gas chromatography-mass spectrometry by the method outlined by Nagy et al. (10). The procedure developed for carambola essence characterization was similar to that used for yellow passion fruit essence.

Yellow Passion Fruit Essence Evaluation

Figure 3 represents a capillary gas chromatogram of aqueous phase yellow passion fruit essence. One hundred eleven compounds were detected, but only 41 components were definitely identified. Esters were present in abundance as manifested by methyl acetate, ethyl acetate, methyl butanoate, ethyl butanoate, butyl butanoate, ethyl hexanoate, hexyl acetate, hexyl butanoate, ethyl octanoate and hexyl hepanoate. In addition, yellow passion fruit essence contained high levels of alcohols, namely, aliphatic C_1-C_8 alcohols (linear, secondary, unsaturated, iso-and anteiso-branched), and terpinyl alcohols (linalool, terpinen-4-ol, -terpineol, nerol and geraniol). Yellow passion fruit aqueous essence isolated from the TASTE essence recovery unit imparted an aroma characterized as floral and estery, with a sweet licorice-like note. A slight unpleasant sulfur topnote was detected by some aroma panelists.

The essence prepared from yellow passion fruit by our TASTE essence recovery unit has a compositional pattern different from essence prepared by traditional laboratory procedures. Those procedures generally entail varying degrees of solvent extraction, simultaneous distillation-extraction and/or headspace analysis. Laboratory procedures produce a total essence fraction, whereas the TASTE essence recovery unit separates essence into an aqueous phase and an oil phase.

By laboratory preparation, Casmir et al. (11, 12) detected over 300 volatile flavorants in passion fruit and identified 22 components of this mixture as possessing passion fruit flavor. Esters (aliphatic, aromatic, terpenoidic) were the most abundant class of volatiles, followed by C_{13} norterpenoids and monoterpenoids. Winter et al. (13) and Engel and Tressl (14) indicated that the sulfur-containing compounds, especially 3-methylthiohexanol and 2-methyl-4-propyl-1,3-oxathianes, were considered key flavor components of yellow passion fruit. Engel and Tressl (15) reported that nerol, linalool, geraniol

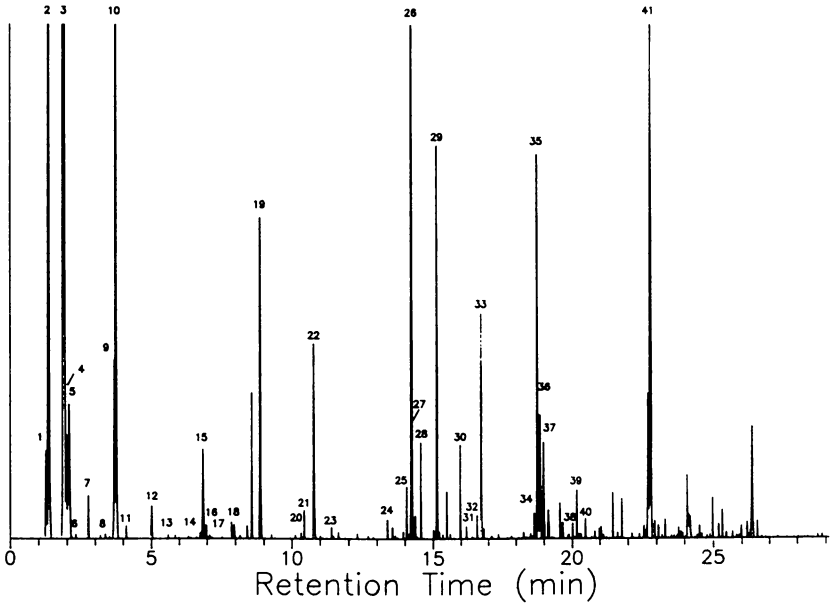

Figure 3. Capillary gas chromatogram of yellow passion fruit aqueous phase essence. Components identified: 1) acetaldehyde, 2) methanol, 3) ethanol, 4) acetone, 5) 2-propanol, 6) methyl acetate, 7) 1-propanol, 8) 2-butanol, 9) 2-methyl-3-buten-2-ol, 10) ethyl acetate, 11) 2-methyl-1-propanol, 12) 1-butanol, 13) 1-penten-3-ol, 14) methyl butanoate, 15) acetal , 16) 3-methyl-1-butanol, 17) 2-methyl-1-butanol, 18) 1-pentanol, 19) ethyl butanoate, 20) trans-2-hexenal, 21) cis-3-hexen-1-ol, 22) 1-hexanol, 23) 2-heptanol, 24) 1-heptanol, 25) butyl butanoate, 26) ethyl hexanoate, 27) octanal, 28) hexyl acetate, 29) limonene, 30) 1-octanol, 31) cis-linalool oxide, 32) trans-linalool oxide, 33) linalool, 34) terpinen-4-ol, 35) hexyl butanoate, 36) ethyl octanoate, 37) α-terpineol, 38) nerol, 39) carvone, 40) geraniol, 41) hexyl hexanoate

SOURCE: Permission granted by Dr. Norman Childers of the Florida State Horticultural Society, Proc. Fla. State Hortic. Soc. 104:51-54 (1991)

and α-terpineol were not present in yellow passion fruits in free form, but were present in bound, glycosidic forms. Thermal treatment of passion fruit pulp at pH 3.0 resulted in increased concentrations of a series of monoterpenes, alcohols and oxides.

Engel and Tressl (15) noted that a pool of nonvolatile polar precursor compounds ("covered flavor") exist in passionfruit. This reservoir of nonvolatile compounds predominately consist of the glycosides of monoterpene alcohols as well as hydroxylated linalool derivatives. The presence of bound flavor compounds may explain the difficulty in comparing aroma profiles derived from the same cultivar, namely, yellow passion fruit. Thermal treatments, either during commercial processing or during laboratory preparation, strongly influence the type and amounts of aroma compounds detected. Yellow passion fruit essence is a mixture derived from (a) natural, unbound flavorants, (b) flavorants released from bound forms by juice acidity and thermal treatments and (c) compounds derived from acid-catalyzed hydration-dehydration reactions and by other type transformations.

Discussion

Total production of tropical fruits in Florida is small when compared to citrus production. Whereas Florida processing plants are designed to handle citrus fruit volumes of about 200,000 to greater than 1 million tons over a 6-month period, this capacity is far in excess to what is needed to process Florida's tropical fruit. Currently, the challenge is to adapt existing citrus processing equipment to handle smaller and diverse fruit loads. To this end, the FMC citrus juice extractor and finisher were adapted for extraction of passion fruit juice (9), and a food chopper and FMC Model 35 finisher were adapted to extract juice from carambola fruit (10). The TASTE citrus evaporator located at CREC in Lake Alfred is a pilot plant unit that can concentrate small volumes of juice. Large capacity, commercial TASTE citrus evaporators would be too inefficient in handling small tropical fruit loads. Therefore, research and development are needed to develop smaller, commercial type units.

An important value-added byproduct of fruit juice processing (citrus and tropical fruits) is fruit essence. The carambola and yellow passion fruit essences produced by our TASTE evaporator were of excellent quality. The potential use of these flavor fractions in foods, beverages and other consumer products appears promising.

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Chapter 6

Volatile Compounds Affecting Kiwifruit Flavor

Harry Young, Margaret Stec, Vivienne J. Paterson, Kay McMath, and Rod Ball

Horticulture and Food Research Institute of New Zealand, Mount Albert Research Centre, Private Bag 92169, Auckland, New Zealand

The flavor of kiwifruit (*actinidia deliciosa*) appears to be a subtle blend of several volatile components, in addition to such non-volatile components such as sugars and acids. The volatile chemical components present and their significance to the flavor of kiwifruit are reviewed. The sweetness of ripe kiwifruit has been shown to be associated with high levels of esters. The results of experiments aimed at determining whether volatile compounds have a causitive effect on the perceived sweetness are presented.

The kiwifruit, *Actinidia chinensis* (A. Chev) Liang et Ferguson var *deliciosa* cv Hayward, is a native of China and is an example of recent success in the domestication and commercialisation of a plant for food. Thirty years ago it was a botanical curiosity. The fruit was first grown commercially in New Zealand but is now a commercial crop in many countries.

At harvest the fruit is very hard and has little characteristic flavour. During ripening the fruit soften considerably, with the production of a large number of volatile compounds. The composition of the flavour volatiles has been the subject of several studies (1,2,3,4), with over 80 components identified.

The main compounds found include methyl and ethyl butanoate, hexanal, Z- and E-hex-2-enal, hexanol, Z- and E-hex-3-enol, methyl benzoate (Table 1). The flavour of kiwifruit is not dominated by a character impact compound, but appears to be a subtle combination of several components.

Unlike some of the other more traditional fruit, e.g. apples and oranges, the levels of the flavour volatiles undergo tremendous changes as the fruit ripens and on storage (5,6). There are large variations in levels of compounds between individual fruit in any one batch.

One of our objectives in studying the flavour of kiwifruit is to be able to identify the compound or compounds which make a substantial contribution to the flavour of the fruit. Although the sugars and non-volatile acids have a major role to play in the flavour of the fruit, this study will focus on the volatile flavour

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Table 1. The major volatile flavour compounds found in kiwifruit

	μg/100gfw		μg/100gfw
Ethyl acetate	60-260	Z-Hex-2-enal	70-30
Methyl butanoate	440-1800	E-Hex-2-enal	2380-1000
Ethyl butanoate	300-1200	Hexanol	21
Hexanal	320-80	E-Hex-2-enol	50-40
Pent-1-en-3-ol	10-6	Methyl benzoate	110-140
Methyl hexanoate	7-9		

compounds which affect the sweet attributes. This knowledge is of utmost importance in developing new varieties with improved flavour and other qualities, and in developing processed kiwifruit products, e.g a kiwifruit juice, with good flavour quality. At present the flavour of processed products leaves a lot to be desired.

Methods

Headspace Analysis. Headspace was carried out as described in Young and Paterson (5). The sample was held in a water bath at 30 °C and the headspace was swept with nitrogen gas at 24 ml/min for 30 min.

XAD-2 Stripping of Volatiles. Juice was prepared from fruit of average fruit firmness 0.5-0.6 kgf and soluble solids concentration 12.9 °Brix to give a juice with 13.1 °Brix using the method of Heatherbell *et al* (7). The juice was stored at -20 °C until required. Amberlite XAD-2 (160 g) was packed into a column (155 mm x 47 mm) and washed with 3 bed volumn of acetone, 3N HCl and finally washed with purified water until Cl^- free. Juice (6.8L) was passed down the column.

Partial Stripping of Volatiles. The juice was prepared as above but without any heat treatment after pressing. The freshly pressed juice had a soluble solids concentration of 13.8 °Brix and was immediately passed through an Alfa-Laval Centritherm Evaporator model CT1B-2 operated at 35 °C under reduced pressure to remove a portion of the volatile aroma components together with some of the water. Soluble solids concentration of the final juice was 18.4 °Brix. The juice was stored at -20 °C until the day of the evaluation when it was thawed and diluted to 12 °Brix.

Experimental Design. In order to keep the number of samples to a manageable number, a fractional multifactorial design was used. This was obtained by taking a suitable one third fraction of the complete factorial design of 729 samples and subdividing this one third fraction into blocks of size of 3. The resulting design was *resolution V* (8) for treatment factors. This gave *unbiased estimates* of main effects and second order interactions, assuming no higher order interactions. Main effects and second order effects were partially confounded with panellist in a balanced way with an efficiency of 33%.

Sensory. The test compounds were dissolved in 5 ml of ethanol and made up to a final concentration with water (*ca* 5 ml) so that 50 μl of the solution contained the appropiate amount to achieve the desired levels in the sensory sample. Samples were made up in individual plastic cups fitted with lids immediately prior to each sensory session. Each sample was made up with 30 ml of the juice base and the appropiate test solutions. Fourteen panellists were used in 2 sessions (morning and afternoon) over 3 consecutive days. At each session panellists evaluated 3 coded samples with the target compounds at levels according to the fractional multifactorial design. The Sweet Flavour, Sweet Aroma, Acidic Flavour and Acidic Aroma attributes were measured using a 0 to 9 point scale (0 = none and 9 = extreme).

Results and Discussion

In GC-Olfactory studies (9) the components which showed the most kiwifruit character were E-hex-2-enal and the butanoate esters, with some contribution from the C_6 alcohols. However we have difficulties in duplicating the kiwifruit flavour with simple mixtures of these chemicals.

Most of the chemical investigations into the flavour of kiwifruit have been done on bulk samples of a large number of fruit. Thus differences between individual fruit will not be detectable. However for practical reasons sensory testing is usually carried out using individual fruit. Hence any relationship between chemical components and sensory attributes will be masked. As mentioned earlier, there are tremendous variations between individual fruit due to the extreme sensitivity of the kiwifruit flavour to the degree of ripeness. This inter-fruit variation could be used to our advantage. If the chemical analyses were carried out on individual fruit and the data related back to the sensory data for that fruit we could in fact make use of this variability to look for trends between chemical components and sensory perception (e.g sweet flavour, characteristic kiwifruit flavour). To be able to do this it is essential to have a method that could handle large numbers of samples over a relatively short space of time since sensory experiments usually involve the tasting of a large number of fruit during each session. It also requires good cooperation from colleagues in sensory science. We have been in the fortunate position at HortResearch to have been able to link together flavour chemistry and sensory science research.

The method we have chosen for analysis of the flavour volatiles is headspace sampling using adsorbent traps (on Chromosorb 105), despite the shortcomings of the method (5). It does not give total extraction of the volatile flavour components but the composition of the compounds recovered is probably a better representation, of that "seen" by panellist, than that obtained by more exhaustive extraction methods. With attention to detail a representative profile of the volatile components in a fruit can be obtained. With this method we were able to collect the volatile components from the fruit at the same time as the sensory testing . The loaded traps were stored at -20 °C until they were analyzed over the next few days by GC.

When linear regression analysis was applied to the volatile aroma data few correlations between levels of volatile components and sensory data were found. One of the most promising was between sweetness and ester levels. However there is no reason to assume linear relationships between the intensity of sensory perceptions and the quantity of a given compound. Indeed it is well known that many of the sensory responses to physical measurements are linear only over a relatively small range at best. The use of a statistical procedure that does not impose linear relationships was more successful. Trends were found for several flavour components. The procedure we have employed was Additivity and Variance Stabilizing Transformations, AVAS for short. It should be emphasized that this is an exploratory tool. McMath *et al* (10) have shown that sweetness and acidity in kiwifruit can be predicted by a number of variables, including some of the volatile flavour components.

It is very important to understand that the results from the AVAS procedure are only indicative and the chemical compound selected does not necessarily have a causative effect. Furthermore these statistical results should not be interpreted without reference to the chemistry. Using this technique positive correlations were found between Sweetness and methyl hexanoate, ethyl hexanoate and methyl benzoate, and negative correlations with ethyl acetate, methyl butanoate and Z-hex-2-enal. Ethyl butanoate, quantitatively a major component in the flavour volatiles was not found to correlate with the sweet attributes. It could be that ethyl butanoate was highly correlated with other components, and hence its relationships were effectively masked, or that the level had exceeded the upper limit at which panellists could distinguish differences.

An experiment was designed to test if esters do indeed affect the Sweet Aroma and Flavour, and Acidic Aroma and Flavour. The volatile flavour compounds were stripped from kiwifruit juice using Amberlite XAD-2 resin. This treatment did not significantly affect the sugar, non-volatile acid levels or the mineral content. In this experiment panellists were asked to determine the intensity of Sweet Aroma and Flavour, and Acidic Aroma and Flavour in samples of the base juice to which the selected compounds have been added at various concentrations and combinations according to a fractional multifactorial design to achieve the levels shown in Table 2. The three levels of compounds used were chosen so that the low value was just above detectable levels in training experiments, the mid-range value similar to what is considered to be approximating a ready-to-eat kiwifruit (1) and the high value was in the range for kiwifruit with high levels of esters (i.e. a slightly over-ripe fruit).

Although a relationship between the sweet attributes and ethyl butanoate was not found during the exploratory analysis it was decided to include it as it is one of the major components in kiwifruit flavour volatiles, it has been described as having a kiwifruit character during GC-Olfactory testing and it has a very low sensory threshold.

Table 2. Headspace levels of compounds in stripped juice base experiment

	μg/100g		
	Level 0	Level 1	Level 2
Ethyl acetate	0.4	147.0	293.6
Methyl	0.2	88.8	177.3
Ethyl butanoate	0.2	212.6	425.0
Methyl	0.5	0.9	1.4
Ethyl hexanoate	3.1	6.2	9.3
Methyl benzoate	5.4	10.8	16.2

Only two of the esters, ethyl butanoate and methyl benzoate, showed significant effects. They increased the intensity of Sweet Aroma and Sweet Flavour (Figure 1). The increase in ethyl butanoate between level 1 and level 2 (from 212 to 425μg/100g) increased Sweet Aroma by 7%. Acidic aroma decreased with increasing ethyl butanoate levels (data not shown). The increase of methyl benzoate between level 0 and level 2 (from 5.4 to 16.2 μg/100g) produced an 8% increase in Sweet Aroma.

Ethyl butanoate has been found to contribute to the sweet flavour of peaches (11) while methyl benzoate contributed to the sweet aroma of guava (12).

A further test was carried out using a diluted kiwifruit juice from which only some of the flavour volatiles were removed. Ideally freshly prepared kiwifruit pulp should be used but the homogeneous incorporation of the chemicals would be very difficult to do reliably. Approximately 50% of the volatiles were removed under vacuum from freshly pressed juice. The resultant juice retained the fresh kiwifruit character. However it had to be diluted because the extreme acid nature of kiwifruit juice was sufficient to severely interfere with the tasting ability of the panellists. The levels of the target volatile flavour compounds are listed in Table 3.

Table 3. Headspace levels of compounds in partially stripped juice base experiment

	μg/100ml		
	Level 0	Level 1	Level 2
Methyl butanoate	55.2	84.0	120.8
Ethyl butanoate	208.8	520.3	869.9
Hexanal	7.4	20.7	40.6
E-hex-2-enal	65.8	136.2	236.6
E-Hex-2-enol	14.0	25.6	34.7
Methyl benzoate	-	7.2	17.3

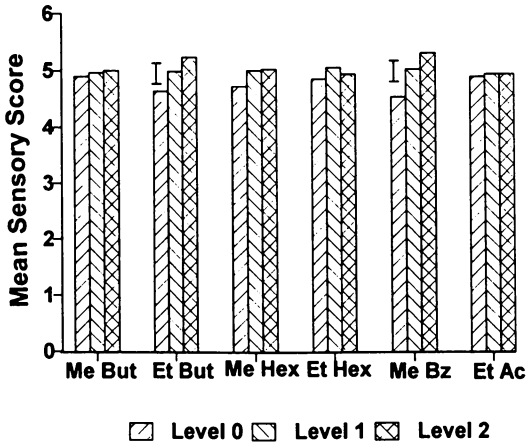

Figure 1a. Mean sensory scores for Sweet Aroma with stripped juice. Me = methyl, Et=ethyl, But = butanoate, Hex = hexanoate, Bz= benzoate, Ac = acetate.

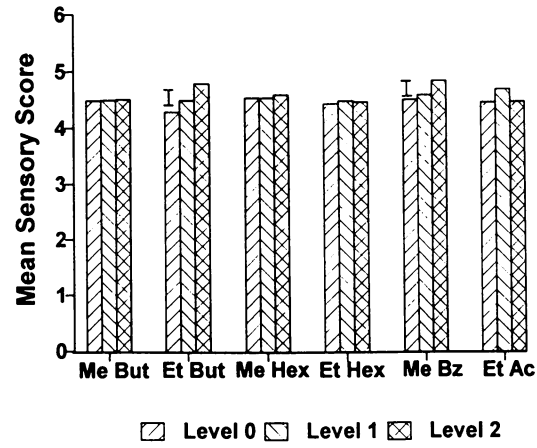

Figure 1b. Mean sensory scores for Sweet Flavour with stripped juice. Me = methyl, Et=ethyl, But = butanoate, Hex = hexanoate, Bz= benzoate, Ac = acetate.

In this latter study the same sensory attributes, Sweet Aroma and Flavour, and Acidic Aroma and Flavour were measured. Analysis of variance was carried out without allowing for interactions since an analysis allowing up to second order interactions did not reveal any interactions between the 6 factors (methyl butanoate, ethyl butanoate, methyl benzoate, hexanal, E-hex-2-enal and E-hex-2-enol). The results for Sweet Aroma and Sweet Flavour are shown in Figure 2.

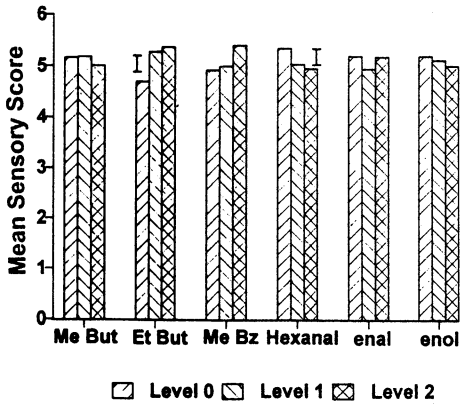

Figure 2a. **Mean sensory scores for Sweet Aroma with partially stripped juice.** Me = methyl, Et = ethyl, Bz = benzoate, enal = E-hex-2-enal, enol = E-hex-2-enol

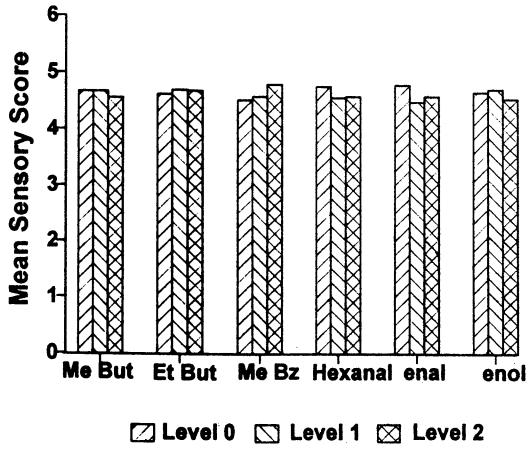

Figure 2b. Mean sensory score for Sweet Flavour with partially stripped juice. Me = methyl, Et = ethyl, Bz = benzoate, enal = E-hex-2-enal, enol = E-hex-2-enol

Ethyl butanoate and hexanal had significant effects on Sweet Aroma. Increasing the level of ethyl butanoate increased Sweet Aroma, although little difference was found between level 1 and level 2. Increasing the level of hexanal decreased Sweet Aroma, the effects at level 1 and level 2 being similar.

In the latter experiment with partially stripped juice significant effects were not found between Sweet Flavour or Acidic Flavour and the six added chemicals. In the experiment using the stripped juice as the base increasing the level of ethyl butanoate increased Sweet Flavour as well as Sweet Aroma. However with the stripped juice base the aroma volatiles were almost completely stripped out. Level 0 in the partially stripped base was similar to level 1 in the experiment using stripped juice base. Hence it could be that the upper threshold for ethyl butanoate, at which panellists could detect differences in Sweet Flavour, has been exceeded. Furthermore the partially stripped juice base contains other esters which are likely to have similar effects as ethyl butanoate, and thus added further complexity to the situation.

In conclusion ethyl butanoate (and other esters) does influence Sweet and Acidic attributes in kiwifruit but the upper threshold at which changes could be detected by panellists are reached at relatively low levels.

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Finally we would also like to thank the organisers for their invitation to take part in this symposium.

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Chapter 7

Detection of Adulterated Fruit Flavors

Dana A. Krueger

Krueger Food Laboratories, Inc., 24 Blackstone Street, Cambridge, MA 02139

Methods employed in the detection of synthetic and artificial flavors are surveyed. GC and HPCL techniques are used to detect substances not naturally presnent in flavors from particular source materials. Capillary GC-MS is used to identify the presence of substances which are not nature-identical. Chiral GC, HPLC and enzymatic techniques are used to identify synthetic racemates substituted for chiral flavoring materials. Carbon 14 analysis is used for identification of petrochemical synthetics. Applications of isotope ratio mass spectrometry and deuterium NMR analysis for differentiating natural and synthetic flavor compounds are discussed.

The marketplace for fruit products, flavors, and syrups has for many years been plagued by the problem of economic adulteration. Economic adulteration is the undeclared substitution for a high valued product of a similar, but inferior and less expensive product. Many natural flavoring materials are much more expensive to produce than corresponding artificial materials, and command much higher prices. This provides a large incentive for this type of adulteration; one which has led to a long history of problems in the flavor trade.

Natural flavors are produced from animal or plant sources by one or more of several "physical" processes. These physical processes include mechanical expression, distillation or solvent extraction. Also included among these processes are the chemical processes of pyrolysis, fermentation and enzymatic reaction. Flavoring materials produced in this manner may properly be called natural.

The regulatory regime in the United States recognizes several types of flavor label descriptions. Natural flavors may be claimed to be natural and from particular defined sources, such as bitter almond oil or apple essence. Alternatively, they may simply be labeled natural flavor, with no reference to the particular flavoring ingredients. All flavoring materials which are not prepared from the approved natural

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sources and processes are properly labeled as artificial. In the European Community, the American artificial category is further subdivided. Synthetic substances are labeled "nature identical" if the same chemical substance occurs in natural foodstuffs, while substances unknown in nature are labeled artificial.

This hierarchy of flavor labeling allows the possibility of three different types of mislabeled or adulterated flavors. A defined source type of flavor would be considered adulterated if it contained additions of any other type of flavor. A natural flavor would be considered adulterated if it contained additions of any synthetic flavoring material. Finally, in European Community countries, a nature identical flavor would be considered adulterated if it contained additions of a synthetic substance of a type which does not occur in nature.

As the producers of fraudulent products become ever more clever in their formulations, substituting artificial for natural flavorings, the quality control analyst requires ever more sophisticated tools for the detection of these products. This article surveys some techniques for the analysis of natural fruit flavorings that have proved useful in detecting economic adulteration. The techniques are illustrated by examples taken from the author's work and from the literature.

Detection of Foreign Components in Defined Source Flavors

Many fruit flavored products are labeled as being flavored solely by the juice or essence of the fruit in question. Such a product would be mislabeled if the flavoring was in fact derived in whole or part from another material. Such adulterations can often be detected by analysis for substances in the product which are not normally found in the fruit.

One of the key flavor impact substances of black currant juice is the sulfur containing substance 4-methoxy-2-methyl-2-mercaptobutane. This substance is responsible for the catty odor of black currants. The essential oil of the buchu plant contains a different substance with a similar aroma. Many imitation black currant flavors utilize buchu oil for the catty note. Buchu oil contains as major constituents isomers of diosphenol, which are not found in black currants. Adulteration of natural black currant products has been detected by capillary gc-ms analysis of the volatile substances. The presence of buchu oil diosphenols is readily detected by this procedure (*1*).

Passion fruit juice is a strongly flavored juice which is very popular in the formulation of nectars and blended fruit juice drinks. Adulteration of passion fruit juice by dilution with orange juice is an occasional problem. Such adulteration can be detected by analysis for substances typical of orange juice. Capillary gc-ms analysis of the volatile substances of passion fruit juice will reveal the presence of significant levels of limonene and other orange oil terpenenes in such adulterated juices. Pure passion fruit contains only traces of limonene (*2*). Examination of the phenolic substances of passion fruit by hplc analysis will also reveal the presence of hesperidin, a substance found in orange juice, but not in passion fruit juice, in such cases. (*3*).

Detection of Components Not Found in Nature

Many artificial flavors are formulated from substances which are not found in natural foodstuffs. Many adulterated flavors can be detected by analysis for such substances.

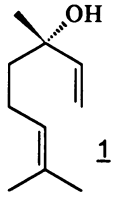
An important flavor impact compound of strawberry juice is 2,5-dimethyl-4-hydroxy-2,3-dihydrofuran-3-one or furaneol. Many formulated strawberry flavors achieve a similar impact through the use of the substituted pyrans maltol and ethyl maltol. Ethyl maltol, which has a particularly intense aroma, is a substance not found in nature. The author, by capillary gc-ms analysis, has found numerous instances of supposedly natural strawberry flavors which contain ethyl maltol, and are thus in fact artificial.

Vanillin is also an important flavor constituent in many natural fruit flavors. The author has found occasional instances by gc-ms analysis where the more intense substance ethyl vanillin has been used in flavors labeled as natural. Ethyl vanillin is also a substance not found in nature.

Detection of Manufacturing Impurities in Synthetic Flavoring Materials

Many artificial flavor chemicals contain traces of impurities which are minor byproducts of their synthesis. These trace substances can be used as markers to indicate the synthetic origin of the substance.

Linalool **1** is a substance which is used in the formulation of many fruit flavors, particularly apricot and peach flavors. Natural linalool is obtained from a number of essential oils. Synthetic linalool is manufactured by the hydrogenation of an intermediate, dehydrolinalool **2**. After partial hydrogenation of **2** to linalool, a small quantity of the fully hydrogenated product, tetrahydolinalool **3** is formed. Synthetic linalool usually contains residual trace quantities of **2** and **3**. Capillary gc-ms analysis of linalool can be used for the detection of **2** and **3**, and can thus be used for the detection of synthetic linalool (4).



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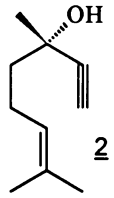

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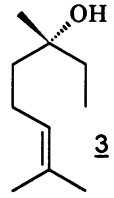
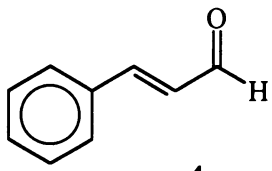


3

Cinnamaldehyde **4** is a substance which is used to add a spicy note to many formulated flavors. Natural cinnamaldehyde is obtained from cassia and cinnamon oils. Synthetic cinnamaldehyde is prepared by the condensation of acetaldeyde with benzaldehyde. During synthesis, a small quantity of a product resulting from the condensation of two molecules of acetaldehyde, 5-phenylpentadienal **5**, is formed.

Capillary gc-ms analysis of cinnamaldehyde can be used to detect traces of **5**, and can thus be used for the detection of synthetic cinnamaldehyde (5).



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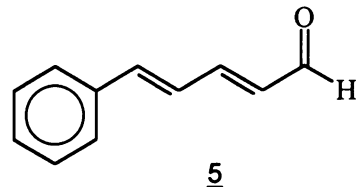

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Determination of Carbon 14 Content

Analysis of the natural radiocarbon content of foodstuffs has been used as a tool to distinguish between natural products and synthetics from fossil fuel sources. Atmospheric CO_2, and natural products derived from it via plant photosynthesis, contains minute quantities of the radioactive isotope ^{14}C. Since ^{14}C has a radioactive half-life of approximately 6000 years, carbon from fossil fuel sources which has been in place for millions of years is essentially devoid of ^{14}C, it having long since decayed away. Therefore ^{14}C analysis can be used as a sensitive indicator of carbon from fossil fuel sources, and consequently of many synthetic flavor chemicals.

The use of ^{14}C analysis for the detection of synthetic flavoring materials was first suggested for vinegar in 1952 (6). ^{14}C content is determined by radiation counting of a suitable derivative using a liquid scintillation counter or a gas proportional counter. It has since been applied to caffeine (7), cinnamaldehyde (8), and other flavor chemicals.

Benzaldehyde is the major flavor impact compound of cherry juice; natural benzaldehyde is used in the formulation of natural cherry flavors. The author showed that ^{14}C analysis can be used to differentiate natural from synthetic benzaldehyde (9).

Benzaldehyde Source	**^{14}C Activity (dpm/gC)**
ex Apricot Kernels	15.7
Synthetic	<0.5

Ethyl butyrate is an important flavor compound found in a variety of natural fruit juices and essences. Natural ethyl butyrate is also produced by fermentation processes. Synthetic ethyl butyrate is produced from petrochemical derived ethanol and butyric acid. The author showed that ^{14}C analysis can be used to differentiate natural from synthetic ethyl butyrate (10).

Ethyl Butyrate Source	**^{14}C Activity (dpm/gC)**
ex Orange Juice	16.5
ex Fermentation	17.5
Synthetic	<0.5

Some synthetic substances are only partially composed of petrochemical carbon. Natural maltol is produced from pyrolysis of birch or larch wood. Synthetic maltol is produced from the condensation of kojic acid, prepared by fermentation, and petrochemical formaldehyde. Therefore, five of six carbons of synthetic maltol derive from natural sources while one derives from petrochemical source. The authors have found that ^{14}C analysis of maltols results in two clusters of results as shown in the following table.

Maltol Type	^{14}C (% of current year value)
Group 1	98-112
Group 2	84-88
Natural (theory)	100
Synthetic (theory)	83

Determination of Carbon Stable Isotope Ratio

A second important category of isotopic analysis of flavors is carbon 13 analysis. Natural variations in the ratio of $^{13}C/^{12}C$ have in recent years been useful in detecting adulterated flavoring materials.

Carbon 13 is a rare isotope of carbon with a natural abundance of approximately 1 atom percent. In terrestrial plant tissue, the principal source of variations in $^{13}C/^{12}C$ ratios is the different photosynthetic pathways for CO_2 fixation. Plants fix CO_2 by one of three pathways: the Calvin cycle pathway (C3), the Hatch-Slack pathway (C4) and the Crassulacean Acid Metabolism pathway (CAM). It is observed that C3 photosynthesis has a very large isotope effect associated with carbon dioxide fixation, while C4 photosynthesis involves only a small degree of carbon isotope fractionation. $^{13}C/^{12}C$ ratios for most plant tissues tend to be clustered in two ranges associated with the C3 and C4 pathways. CAM plants, which are relatively rare, give $^{13}C/^{12}C$ ratios intermediate between the C3 and C4 ranges. Other, smaller variations in $^{13}C/^{12}C$ ratio occur as a result of enzymatic reactions associated with the synthesis of various molecules in the plant tissue.

$^{13}C/^{12}C$ ratios are normally measured by ratio mass spectrometry. The sample is combusted to CO_2, and the ratio of the ions m/e 45 ($^{13}C^{16}O_2$) and m/e 44 ($^{12}C^{16}O_2$) are measured. This sample ratio is compared with that of a reference gas of known $^{13}C/^{12}C$ ratio. In this way, very small differences from the standard ratio may be precisely measured. $^{13}C/^{12}C$ ratios are reported as parts per thousand (permil, o/oo) difference between the sample ratio and the ratio of an arbitrary standard Pee Dee Belemnite limestone (PDB) according to the formula:

$$\delta^{13}C = 1000\left[\frac{^{13}C}{^{12}C}sample \Big/ \frac{^{13}C}{^{12}C}PDB - 1\right]$$

Adulteration of fruit juices requires the addition of sugars to maintain the soluble solids level. Most fruits are from C3 plants, while corn and sugar cane are both C4 plants. The $^{13}C/^{12}C$ ratio of fruit juices can be used to detect addition of cane sugar and corn syrup to fruit juices. The author and his colleagues have reported on the $^{13}C/^{12}C$ ratio of various fruits, toward the goal of detecting such additions (11-14). Some previously unpublished results from apple, orange and strawberry juice are reported in the following table.

Fruit Juice	$\delta^{13}C$ (‰ PDB)	# of Samples
Apple Juice	-25.3 ± 0.5	484
Orange Juice (Florida)	-25.5 ± 0.3	72
Grapefruit Juice	-26.1 ± 0.6	29
Strawberry Juice	-24.3 ± 0.8	23
Cane and Corn Sugars	-10 to -12	

Ethyl butyrate from orange juice has been shown by the author to be distinguishable from ethyl butyrate prepared by fermentation through the use of $^{13}C/^{12}C$ ratios (10).

Ethyl Butyrate Source	$\delta^{13}C$ (‰ PDB)
Orange Juice	-29.6
Fermentation	-14.7

One new technique has been developed which may prove of great utility in the evaluation of flavor and essence materials. An isotope ratio mass spectrometer has been configured as a gas chromatographic detector. The $^{13}C/^{12}C$ ratio of minute quantities of volatile substances can be measured as they emerge sequentially from the gas chromatograph outlet. This can allow the detection of added flavoring materials in fruit juice essences (15).

Determination of Deuterium Content

The relative abundance of the rare hydrogen isotope deuterium has proved a useful indicator in a number of flavor adulteration problems. Hydrogen is a very light element and its isotopes are prone to a wide variety of fractionations due to various chemical reactions and physical processes. Differences in deuterium content in materials from different sources can be used to determine the source of the material.

The total deuterium content of flavoring materials is ordinarily determined by ratio mass spectrometry, similarly to carbon stable isotope ratio analysis. The sample is combusted, the combustion water is recovered and converted to hydrogen gas. The D/H ratio of the gas is then determined by mass spectrometry and reported as ‰

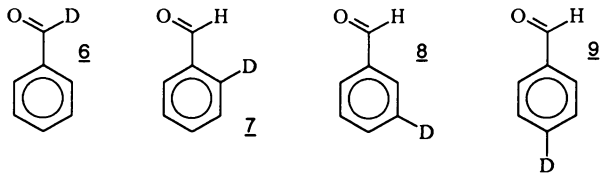
difference from that of an arbitrary standard called standard mean ocean water (SMOW).

The author and others have shown that the D/H ratio of benzaldehyde and cinnamaldehyde provides a useful way of distinguishing natural benzaldehyde and cassia from synthetic equivalents. Synthetic benzaldehyde prepared by catalytic air oxidation of toluene is highly enriched in deuterium content compared to the starting toluene and to natural benzaldehyde from bitter almond kernel oil. Since synthetic cinnamaldehyde is prepared from benzaldehyde, a similar effect is observed in cinnamaldehyde relative to natural cassia oil. Both synthetic benzaldehyde and cinnamaldehyde have D/H ratios approximately 400-600 ‰ more positive than bitter almond oil or cassia oil (16-18).

Benzaldehyde Source	**δD (‰ SMOW)**
Natural	-105 ± 15
Synthetic Type I	+380 ± 60
Synthetic Type II	-40

High resolution deuterium NMR has emerged as a useful tool in detecting flavor adulteration. Deuterium NMR allows the determination of the intramolecular distribution of deuterium. Since deuterium is not uniformly distributed within a molecule, the relative abundance at different molecular sites can provide useful information regarding the origin of substances.

The author worked with colleagues to demonstrate that deuterium NMR can be used to differentiate benzaldehyde from a variety of natural and artificial sources. The table shows the relative abundance of deuterium at the various molecular sites of benzaldehyde from various sources. Significant differences are observed among all of the types of benzaldehyde examined (19).



Benzaldehyde Source	**Relative ^{2}H Abundance**		
	6	**7**	**8+9**
Bitter Almond Oil	0.93	1.87	3.20
ex Cassia Oil	0.92	2.18	2.90
Synthetic Type I	3.27	1.11	1.62
Synthetic Type II	1.02	2.00	2.99
Random	1.00	2.00	3.00

Determination of Enantiomer Ratios of Chiral Compounds

Many flavor compounds are chiral molecules. Frequently the distribution of enantiomers of natural flavor substances is unequal, with one enantiomer predominating. Most synthetic flavor substances consist of equal amounts of both enantiomers. Additions of synthetic flavors to chiral natural flavors can often be detected by chiral analysis.

1,4-decanolide (γ-decalactone) is an important flavor component of peach and other fruit juice flavors. Peach 1,4-decanolide has been shown to consist of approximately 90% (R) and 10% (S) isomer, whereas the synthetic substance is racemic. Analysis of a suitable diasteriomeric derivative allows for the detection of additions of synthetic 1,4-decanolide (20).

Ethyl-2-methylbutyrate is an important flavor impact compound in apple juice flavor. Apple juice ethyl-2-methylbutyrate has been shown to be primarily the (S) isomer, while the synthetic form is racemic. Analysis of apple essence by capillary gc analysis on modified cyclodextrin chiral capillary columns can detect additions of synthetic ethyl-2-methylbutyrate through examination of the enantiomer ratio (21).

In many fruit juices, such as apple and pear juice, the predominant organic acid is malic acid. Natural fruit malic acid consists entirely of the L- isomer. Evaluations for addition of synthetic malic acid have been performed by chiral hplc in apple juice (22) and enzymatic analysis in pineapple juice (14), examining for traces of added D-malic acid .

Conclusion

To conclude, while economic adulteration continues to be a real problem in the food industry ever more powerful methods for detecting it continue to be developed. The commercial fraud is finding his job more and more difficult and more and more expensive. The problem will probably never be completely eliminated, but new analytical breakthroughs promise to reduce it to a relatively small scale.

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Chapter 8

Multisite and Multicomponent Approach for the Stable Isotope Analysis of Aromas and Essential Oils

Gérard J. Martin

Université de Nantes, Centre National de la Recherche Scientifique, 2 rue de la Houssinière, 44073 Nantes Cédex 03, France

The application of Site-specific Natural Isotope Fractionation studied by ^{2}H-NMR to the authentication of flavours and fragrances is illustrated in the case of terpenoid metabolites issued from the mevalonic pathway. NMR spectroscopy offers a multi-site approach to the study of the isotopic fractionation which occurs between the different isotopomers of a given chemical species, geraniol, linalool, (and their esters), citral limonene, pinenes, carvone, camphor, menthone.

An isotopic genealogy may be established between the parent compounds and their descendants. The NMR multi-site procedure is refined by the multi-component approach provided by the coupling of ^{13}C Isotope Ratio Mass Spectrometry with a gas chromatograph and a combustion interface (GC-C-IRMS).

Indeed, for a given series of plants, it is possible to study both the ^{2}H isotopomers of the major constituents and the overall ^{13}C contents of most of the components of the essential oil considered. The $\delta^{13}C$ values given by GC-C-IRMS must be weighted by a precise mass-balance of the oil. Typical examples are presented for carvones, linalools and limonenes found in plants of different origins. It is shown that the carbon fractionation of a metabolite depends closely on the conversion rate of its precursor.

The early applications to flavour and fragrances of Site-specific Natural Isotopic Fractionation studied by Nuclear Magnetic Resonance (SNIF-NMR) was mainly devoted to the products issued from the shikimic bio-genesis pathway. In the 1982-1984, primary interest concerned anethole which is extensively used in the preparation of popular french liquors (1,2,3).

Vanillin was the second important aroma to be studied in Nantes (4,5,6) during a three years period (1985-1987) and a systematic study of aromas containing a benzenic structure (benzaldehyde, cinnamaldehyde, estragol, rasperone...) completed the investigation of the shikimic acid pathway (7,8,10). Dedicated ^{2}H-NMR methodologies were developed from an analytical point of view for the authentication of aromas and perfumes containing these benzenic molecules (anis, cherry, cinnamon, dill....). Excepted two short studies of pinenes and limonenes (11,12), no systematic

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investigation of terpenes by deuterium NMR was carried out before 1990. The monoterpenes are interesting molecules from both a mechanistic and analytical point of view.Indeed, acyclic, cyclic- and bicyclic- monoterpenes are key compounds in the understandingof the bio-synthetic pathways derived from the mevalonic acid metabolism and isotopic fractionation at the natural abundance is a favoured tool for studying the molecular mechanisms linked to monoterpenes biosynthesis. However the isotopic fingerprint of a terpene is also a very efficient instrument for origin authentication purposes and the case of linalool containing essential oils was thoroughly studied by ^{2}H-NMR (13,14) in the frame of a more comprehensive study of terpenes (15,16).

Generally speaking, SNIF-NMR leads to unambiguous information concerning the natural status of a chemical species in an aroma or perfume and in many cases, this technique is able to quantify the degree of adulteration of a product by its synthetic analogue. In specific cases, the botanical origin of a molecule has significant influence on its isotopic fingerprint, irrespectively of its C_3 or C_4 photosynthetic metabolism. For example, anethole extracted from star anise has a different ^{2}H distribution than anethole from fennel, both plants having a C_3 metabolism but growing in rather different environmental conditions.

However, more specific isotopic information which are specifically related to the physiology of the plants would be required in order to make definitive diagnosis about their botanical origin in most of the commercial essential oils found in the market. The coupling of gas chromatography, with a combustion interface and an isotope ratio mass spectrometer (GC-C-IRMS) is expected to give a significant contribution for solving the problem, since the ^{13}C isotope ratio pattern of the different components of an essential oil is directly related to the oil composition. The GC-C-IRMS technique offers the possibility of determining the isotope ratios of major compounds such as linalool and minor constituents such as geranyl acetate may be studied in the same way without any prior separation

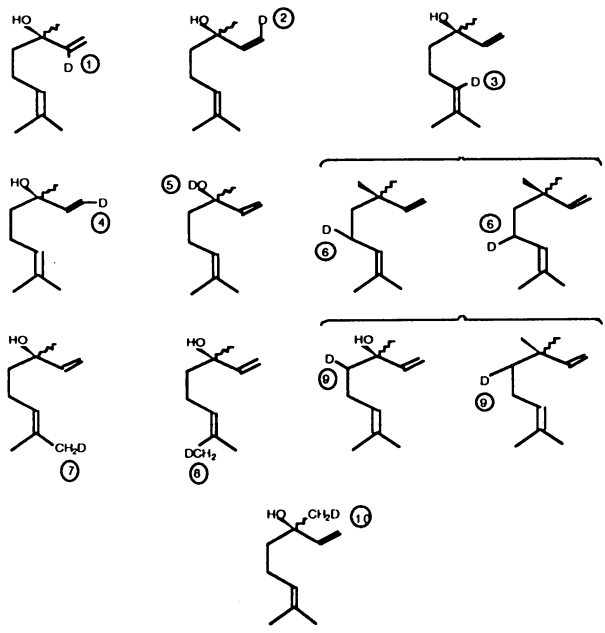
COMPONENT	%$_{w/w}$	δ^{13}C ‰
LINALYLACETATE	34	-27.5
LIMONENE	33	-26.6
LINALOOL	16	-27.3
β-PINENE	6	-25.9
γ-TERPINENE	5	-28.1
β-MYRCENE	1	-26.8
NERYL ACETATE	0.5	-31.8
GERANYL ACETATE	0.5	-33.0

Then the purpose of this talk is to present results concerning the combination of deuterium NMR and on-line ^{13}C GC-IRMS to the authentication of essential oils and aroma extracts. First, the performances of both techniques will be illustrated by typical examples and the refinement of SNIF-NMR by GC-C-IRMS will be presented in the case of the determination of the botanical origin of an essential oil.

Materials and Methods

Materials studied : The two techniques used present indeed interesting complementarities. First of all two elements, H and C are considered and secondly, a relatively great number of species, about 30 to 35, can be studied. Acyclic monoterpenes (Myrcene, Ocimenes, citronellol (and esters), geraniol, nerol, citronellal, geranial and neral), Monocyclic (cymene, limonene, phellandrenes, terpinenes, menthol, terpineols- and esters-, carvone, menthone, piperitone and pulegone) and bicyclic (borneol -and esters-, camphene, pinenes, cineole, camphor and thujone) compounds are usually fond in most of the essential oils.

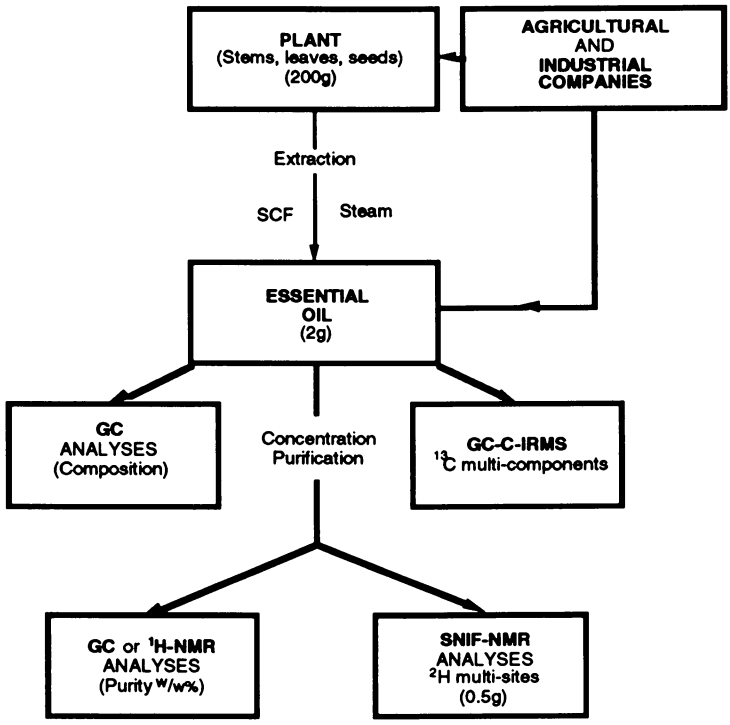
The products considered in this study were either given as complementary samples by industrial firms which are kindly acknowledged, or purchased as chemicals or undefined oils by commercial firms.



However, for each kind of oil studied, were also extracted in the laboratory authentic samples from well defined plants, picked up in situ or purchased by specialized agricultural companies.

Methods :

Treatment of the samples :The general procedure used to prepare a sample for isotopic analysis is presented in the following scheme :



Typically 1 or 2g of oil are required for NMR and MS determinations. A precise and accurate composition of the oils should also be carried out by GC, - the structural identification is done by the conventional GC-MS procedure, supported by a suitable data-bank. For ^{2}H (and ^{13}C) NMR a concentration and purification of one or two major compounds of the oil is also required and the purity must be determined by GC and ^{1}H-NMR.

Isotopic determinations: The main steps of a deuterium NMR determination for obtaining reliable isotopic values are summarized below :

i)- Assignment of signals to isotopomers by ^{1}H- and ^{13}C-NMR : this task is not trivial since a number ^{1}H-NMR spectra of terpenes need yet to be correctly assigned, namely by 2D-NMR techniques

ii)- Recording ^{2}H-NMR spectra: Typically two field strengths were available (BRUKER, 61.4 and 76.5 MHz) for the determination of quantitative ^{2}H-spectra

iii)- Quantification of ^{2}H-signals by automatic curve fitting procedures : this step involves a dedicated software designed in Nantes (EUROLISS TM) which eliminates any systematic bias introduced by conventional base line correction and phase adjustment procedures

iv)- Computation of site-specific isotope ratios by the external comparison procedure :

$$(D/H)_i = \frac{f_i}{F_i} (D/H)_{MS}$$

(f_i is the molar ratio of isotopomers obtained from NMR spectra and $(D/H)_{MS}$ is the mean isotope ratio obtained from MS after combustion of the sample with a SIRA 9 VG Instruments mass spectrometer)

The repeatability and reproducibility of the NMR determinations are nearly equivalent as a consequence of the use of the efficient curve fitting procedure, on the other hand the repeatability is a function of signal-to-noise and is typically equal to :

0.5% or 5% for S/N = 150 or 30

The methodology for GC-IRMS of carbon is also summarized as follows :

i)- Chromatographic separation of the components (HP 5890) : a careful chromatographic separation study of the mixture to be analysed by GC-C-IRMS should be carried out before hand

ii)- Structural identification of the components (HP 5771) : the main constituents of the oil (% ≥ 0.5) should also be identified by running a GC-MS spectrum and comporting the Kovats indices of the components. Such a cross-validation procedure leads to unambiguous structural assignment

iii)- Chromatographic quantification of the components (HP 5890) : the precise and accurate composition of the mixture expressed in terms of % w/w is a prerequirement for the GC-C-IRMS approach and this task is carried out according to the internal reference procedure

iv)- Determination of ^{13}C-isotope ratios (Finnigan Delta S) : The separation of the different components is obtained on a capillary column having the same polarity and performances that the column used in step i). After combustion of individual components in an oxygenated furnace and separation of water the MS-determination is performed on CO_2 . As a consequence of the aging of the column the long term reproducibility may differ from the repeatability. Usually, each mixture is analysed in triplicate and the repeatability of 3 injections ≤0.2‰. The accuracy must be checked every day against specific standard calibrated by IRMS and this procedure gives a long term reproducibility better than 0.4 ‰.

Application of ^2H-SNIF-NMR to the Authentication of Essential Oils

The deuterium NMR study of two typical molecular probes carvones and linalools, will illustrate the potentialities and the limits of this technique

The case of carvones is an interesting example to discuss. It is well known that both enantiomers, - the levogyre R and the dextrogyre S - have typical odours of mint and caraway and may be easily differentiated by this way !

However, at least two kinds of levogyre carvones are found in the market, a spearmint carvone and a hemisynthetic carvone produced from limonene.

These two samples of levogyre R carvones are easily differentiated by ^2H-NMR and a typical ^2H-NMR spectrum of carvones recorded at 76.5 MHz is shown on Figure 1.

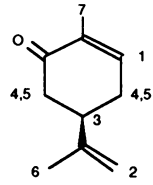
The principal component representation of carvones (Figure 2) demonstrates that the three groups of carvones are easily differentiated using the site-specific isotope ratios measured by ^2H-NMR.

Linalool is an interesting probe, both from the analytical and metabolic point of view since it is found in a great number of essential oils :

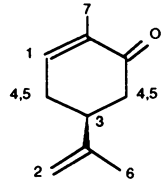
Ho-leaf (85%), Bois de Rose, Coriander (70%), Lavender, Spike Lavender (45%), Bergamot, Petit Grain, Geranium, Clary sage... (<20%)

It is also synthetized from pinenes (via myrcene or pinanol) or from fossil materials (methylheptenone).

The ^2H-NMR spectra of natural (Fig. 6a) and synthetic (Fig. 6b) linalool demonstrate the ability of NMR for establishing the natural status of linalool contained in a product (Fig. 3).



(-)-4(R) carvone



(+)-4(S) carvone

	[SPEARMINT]	[HEMISYNTHETIC]	[CARAWAY]
(D/H) 1	29.2	124.4	23.8
(D/H) 2	147.6	141.8	164.2
(D/H) 3	126.7	92.6	95.4
(D/H) 6	93.5	103.2	99.7
(D/H) 7	109.5	116.3	121.6

It should be kept in mind, that in plants, the relative proportion of dextrogyre and levogyre linalool depends on a number of factors and the optical rotation can not be considered as a safe criterium of origin.

If ^{2}H-NMR is a good technique for distinguishing natural and synthetic linalool, the determination of the botanical origin is a bit more difficult. It will be shown later that the problem can be solved by the joint use of ^{2}H-NMR and GC-IRMS and Fig. 4 presents a bidimensional representation of linalools in the plane of the two main components.

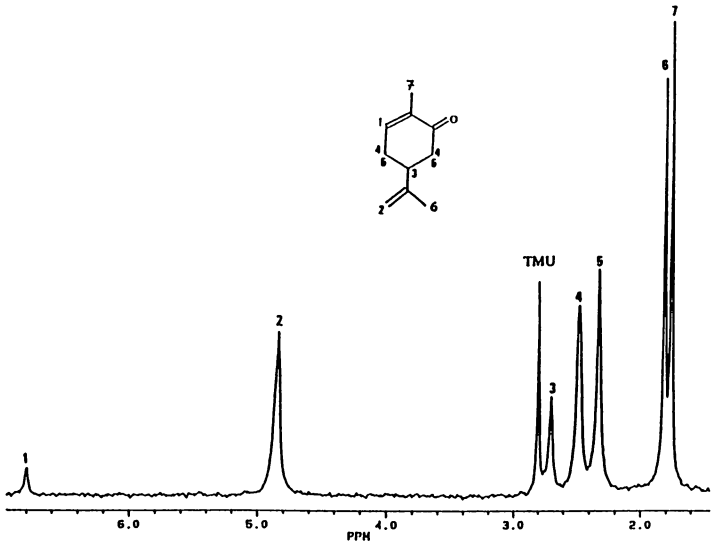


Figure 1. 61.4 MHz ^{2}H-NMR spectrum of (-)4(R) carvone extracted from mint.

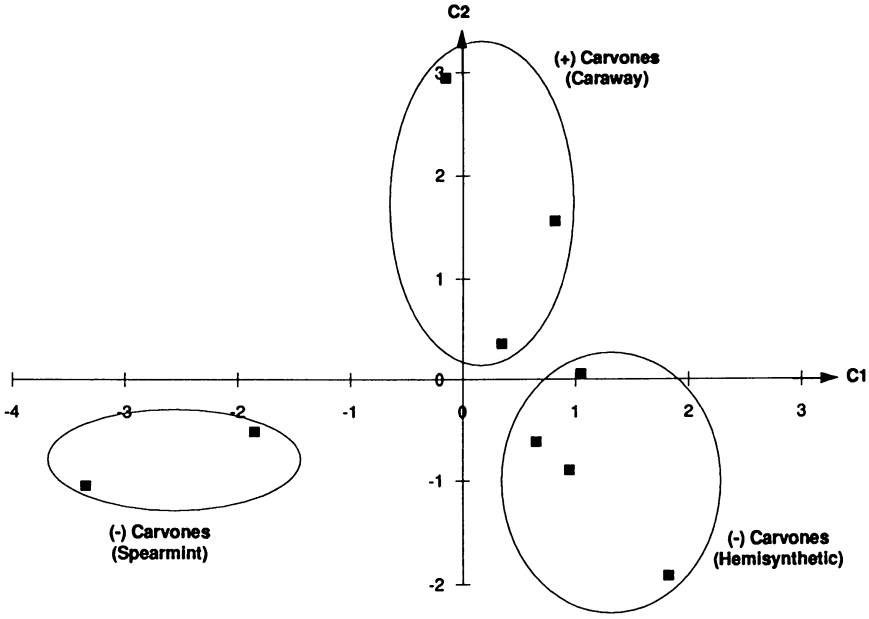


Figure 2. Representation of carvones in the first two components (82%) constructed from seven (D/H) isotope ratios.

Contribution of ^{13}C-GC-IRMS to the study of essential oils containing linalool and limonene

Indeed, linalool is a key compound in terpenes biosynthesis and is found in a number of plants. In the same way, limonene can be considered as the closest cyclic relative of linalool. Both molecules show great differences in composition in the plants, linalool being the most abundant compound but in bergamot oil (Figure 5).

Now, Figure 6 shows the $\delta^{13}C$ values of linalool and limonene measured in the samples from different origins. If we keep in mind that linalool is the major compound in most of the oils but bergamot, we may observe that the $\delta^{13}C$ value of linalool is always higher than that of limonene, but in bergamot. This interesting fact will be tentatively explained later, but the relationship between oil composition an $\delta^{13}C$ distribution in plants is emphasized by the data of Table 2.

Two samples of bergamot oil having different compositions are shown in the table : in sample 1, limonene is the major compound and has a slightly higher $\delta^{13}C$ value than linalool but in sample 2 which has a different geographical origin, limonene and linalool have nearly the same composition but now limonene has a significant smaller $\delta^{13}C$ value than linalool

	Sample 1		Sample 2	
	%	$\delta^{13}C‰$	%	$\delta^{13}C‰$
Linalool	13.4	-26.4	24.3	-27.8
Linalyl acetate	31.2	-26.5	38.5	-28.4
Geranyl acetate	0.4	-30.9	0.2	-33.5
β-myrcene	1.0	-26.0	0.7	-29.0
Limonene	34.8	-26.0	24.4	-28.5
β-pinene	7.3	-25.7	4.5	-27.8

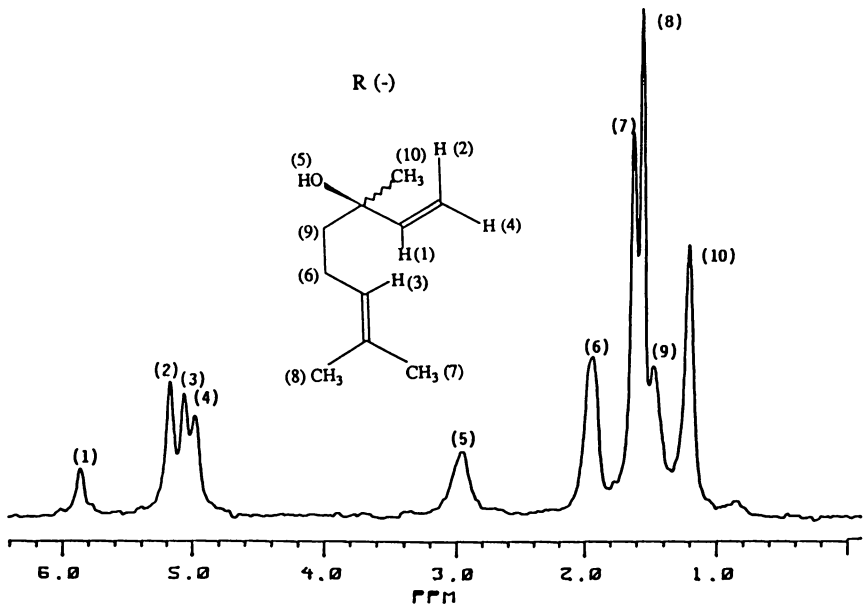


Figure 3a. 61.4 MHz ^{2}H-NMR spectrum of natural linalool.

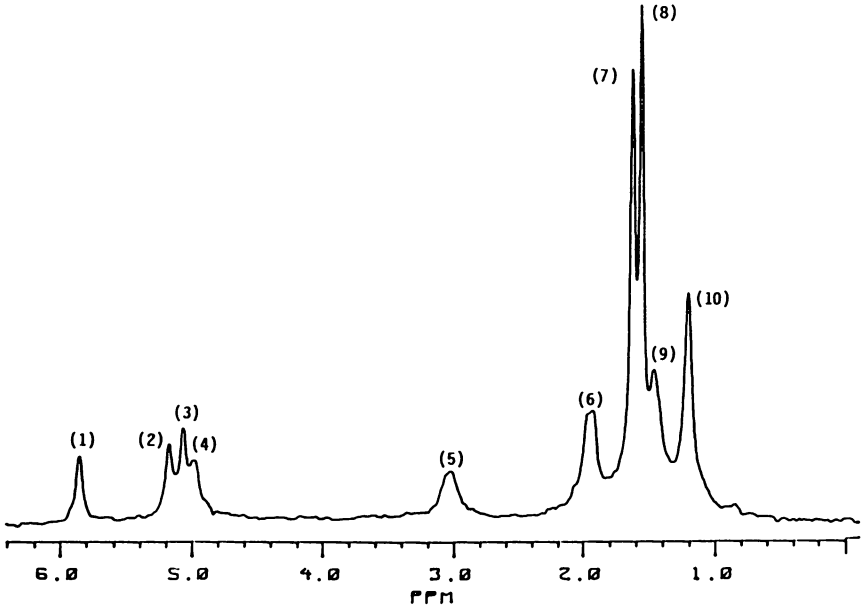


Figure 3b. 61.4 MHz ^{2}H-NMR spectrum of natural and synthetic linalool.

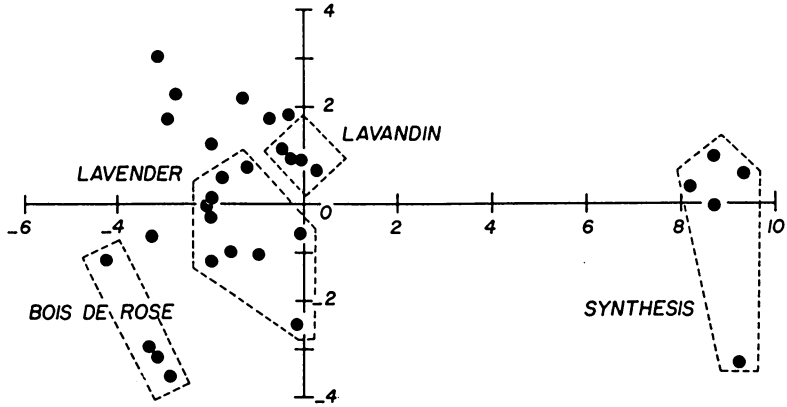


Figure 4. Bidimensional representation of linalools in the plane of the two main components.

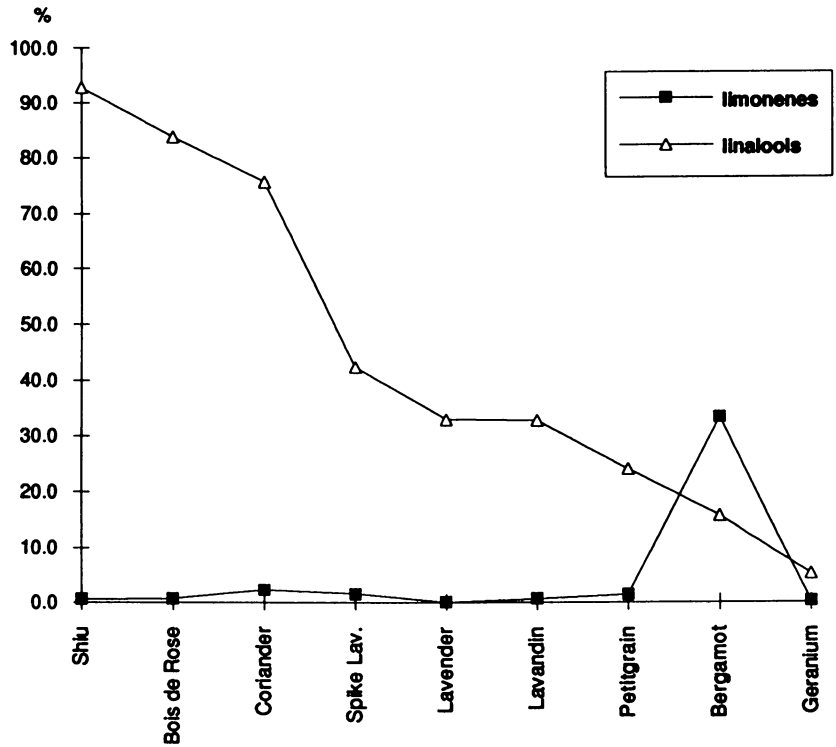


Figure 5. Percentage of linalools and limonenes in different essential oils.

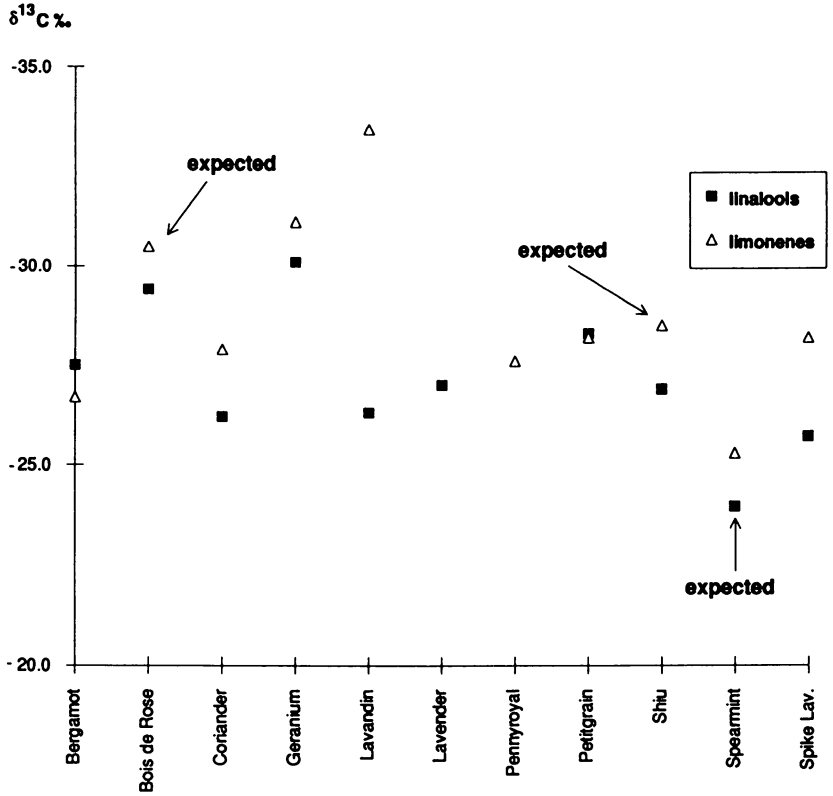


Figure 6. ^{13}C isotope ratios of linalools and limonenes from different origins

The same relative behaviour may be observed if we compare the ^{13}C values of linalool and limonene in plants showing different chemical compositions : and, generally speaking, when comparing two biogenetically related compounds (i.e. the precursor and the product), higher the chemical composition, higher the ^{13}C content.

As a first conclusion it can be stated that -$\delta^{13}C$ values are useless if the relative product composition is not available !

Consider a substrate, i.e. linalool, and the corresponding product, i.e. limonene.

If we assume a direct kinetic isotopic effect ($k_{12} > k_{13}$), the variation of the isotopic fractionation $1/a_s$ and $1/a_p$ is shown on Figure 7. When the conversion rate r_1 in a plant is higher than r_2 of another plant, higher values are observed for R_s/R_{so} and R_p/R_{po}. This simple model may be improved by considering consecutive and concurrent reaction steps and work is in progress to rationalize the whole set of results.

During the monoterpenes biosynthesis, typical reaction steps give characteristic isotopic fingerprints, but they must always be explained in terms of chemical composition.

To conclude, this section it is worth to discuss the influence of the environmental conditions on the $\delta^{13}C$ values of some monoterpenes extracted from plants grown in different regions. The $\delta^{13}C$ values can be related to the water-use-efficiency of the plant, all other factors being kept constant. In Mediterranean regions, the $\delta^{13}C$ values of a given component of a well defined plant species are higher that those of the correspondingly molecules of the same plant grown in humid countries having an Atlantic climate.

Combination of ^{2}H-SNIF-NMR and ^{13}C-GC-IRMS for the authentication of essential oils

The discrimination power of multi-site ^{2}H-NMR expressed in terms of botanical origin may be refined by ^{13}C multi-component analysis results concerning bergamot and petit grain are shown in Figures 8a and 8b.

The site-specific isotope ratios of linalool would lead to a clear cut distinction of natural and synthetic products, and, as far as the botanical origin is concerned, coriander

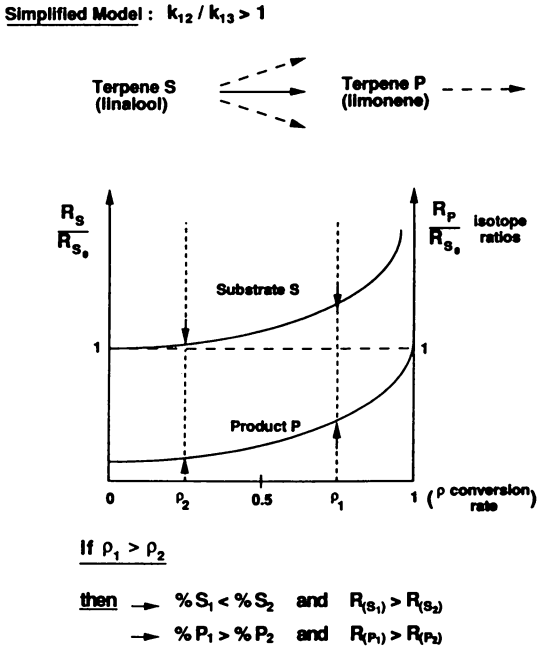


Figure 7. Dependence of the carbon isotopic fractionation on the essential oil composition.

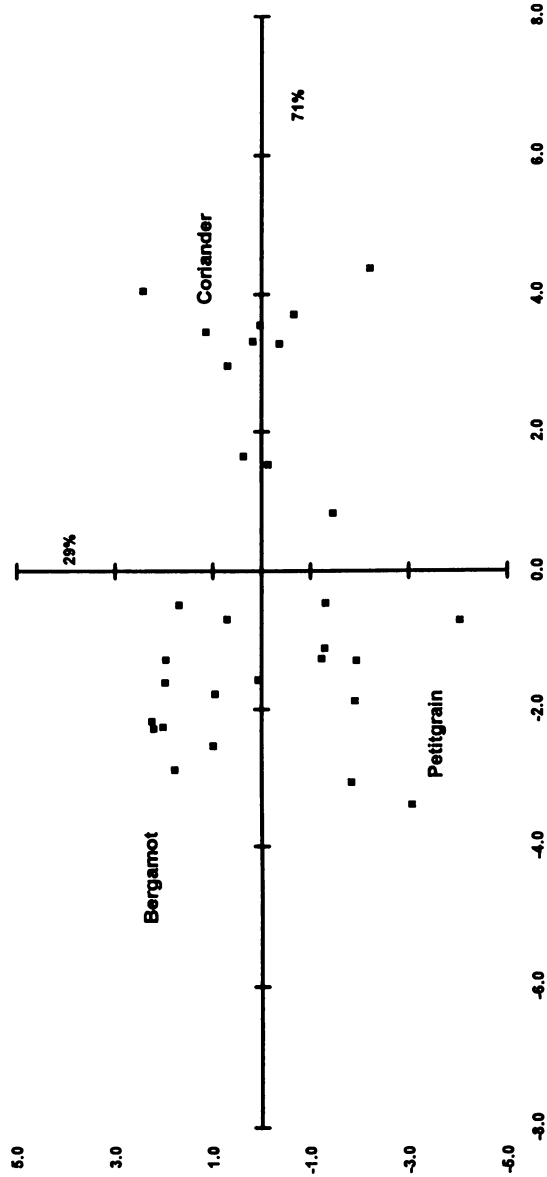


Figure 8a. Authentication of Essential Oils: 5 multi-sites isotope ratios.

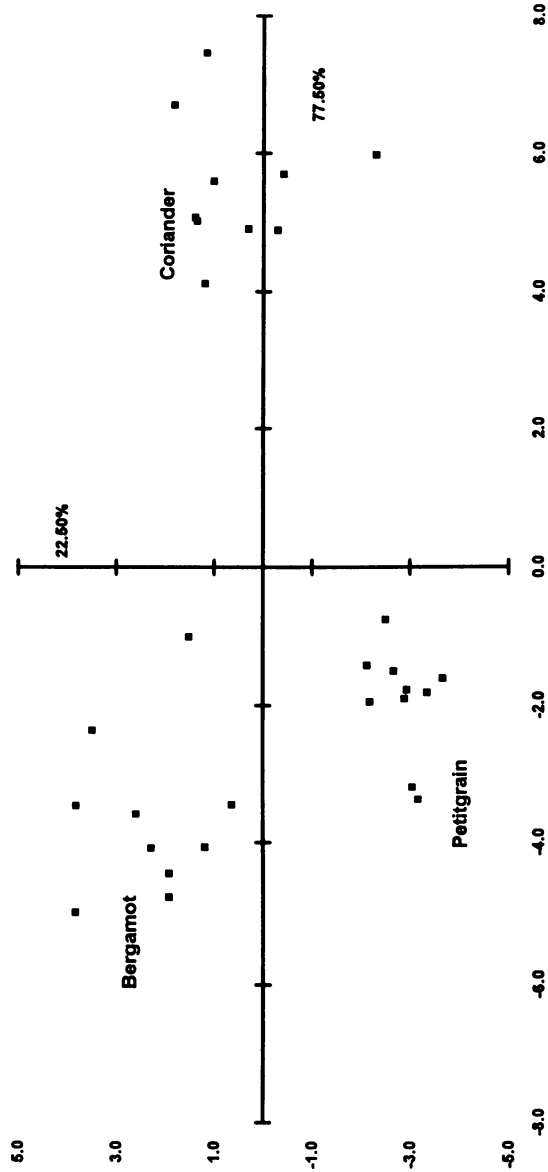


Figure 8b. Authentication of Essential Oils: 5 multi-sites (D/H)i and 4 multi-components 13C isotope ratios.

linalools may be characterized at a high confidence level. There is however a small overlap of the existence domains of bergamot and petit grain linalools (Fig. 8a). If the values of the ^{13}C isotope ratios of 4 characteristic components of the essential oils (b-mycenes, geranylacetate, linalool and limonene) are added to the 5 discriminating $(D/H)_i$ isotope ratios of linalool, (Figure 8b), the bergamot and petit grain essential oils are fully characterized.

Acknowledgments

The ^{2}H-NMR and ^{13}C-GC-IRMS results presented here are taken from the PhD theses of Sophie Hanneguelle and Olivier Breas respectively. Dr. François Fourel and Mm. Françoise Mabon are also kindly thank for their technical assistance. The following firms are also aknowledged : Adrian (F), Apaesa (G), Basf (D), Argeville (F), Berje (USA), Bordas (SP), Bush-Boak-Allen (USA/F), Cavellier (F), Citrus Allied (USA), Firmenich (CH), Givaudan (CH), Hoffmann (CH), Kuraray (J), Laget (F), Munoz-Galvez (SP), Renaud (F), Robertet (F), Rouviere (F), Roure-Bertrand-Dupont (F), Rhone-Poulenc (F), Sanofi (F), SCM-Glicodco (USA).

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Chapter 9

New Methods To Assess Authenticity of Natural Flavors and Essential Oils

A. Mosandl, R. Braunsdorf, G. Bruche, A. Dietrich, U. Hener, V. Karl, T. Köpke, P. Kreis, D. Lehmann, and B. Maas

Institut für Lebensmittelchemie, Biozentrum J. W. Goethe-Universität, Marie-Curie-Strasse 9, D-60439 Frankfurt am Main, Germany

Enantioselective capillary gas chromatography (enantio-cGC) and comparative stable isotope ratio mass spectrometry (IRMS), on-line coupled with cGC, are sophisticated methods in origin specific analysis of volatiles. Scope and limitations of the methods are discussed in view of the genuineness of natural flavors and fragrances.

Pleasant odors enhance the feeling of well-being. Spices and resins from natural sources have been used extensively for perfumery and flavor purposes to enhance product acceptance from the earliest times. Against this background, legal regulations require the differentiation of natural and non-natural substances. Thus, efficient tools are needed in the authenticity regulation of flavors and fragrances.

Site-specific natural isotope fractionation, detected by quantitative ^{2}H-NMR measurements (SNIF-NMR), isotope ratio mass spectrometry (IRMS) as well as enantioselective capillary gas chromatography (enantio-cGC) have been recently introduced as powerful tools to determine genuineness. They have been adopted by the flavor and fragrance industry [1] as part of their quality assurance programs. This paper deals with an overview of our investigations in the use of enantio-cGC as well as IRMS-measurements to determine flavor and fragrance authenticity.

Experimental

Enantioselective Multidimensional Gas Chromatography (enantio-MDGC) Instrumentation. A schematic diagram of enantio-MDGC, is shown in Fig. 1. The design has been well proven in quality assurance and origin control of flavors and fragrances. Shown in the figure is a double-oven system with two independent

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temperature controls, and two detectors (DM1, DM2). A "live switching" coupling piece is used to switch the effluent flow to either the first detector or the chiral column. With optimum pneumatic adjustment of the MDGC system, certain fractions, are selectively transferred onto the chiral main column as they elute from the precolumn (heart-cutting technique).

First, chromatographic conditions of the main chiral column must be carefully optimized. Capillary columns coated with chiral stationary phases of suitable enantioselectivities are used as main columns. Chiral separations are commonly achieved isothermally or with small temperature gradients (low rates of temperature change). Initial temperature of the chiral column is at least 20°C below that of the precolumn. Precolumns are chosen for their ability to offer the most versatility of application, their ability to allow the direct injection of high sample volumes and with respect to the desired time of analysis.

Under optimized operating conditions, uncoated and deactivated restriction capillaries are installed between the injector and precolumn by means of simple press-fit connectors to reduce the carrier gas velocity within the precolumn. Using such a column configuration, suitable pre- and main columns may be easily exchanged and adapted for optimum efficiency [2].

Detection Systems. If optimum chiral separation conditions and high efficiency sample clean-up are properly employed, the first priorities in enantioselective analysis have been achieved. Under such conditions, simple detection systems, such as flame ionization detection (FID) can be used. However, the ideal detector is universal yet selective, sensitive and structurally informative. Mass spectrometry (MS) currently provides the closest realization to this ideal [3]. The combination of multi-dimensional GC with high-resolution MS or mass-selective detectors in the single ion monitoring (SIM) mode is currently the most potent analytical tool in enantioselective analysis of chiral compounds in complex mixtures. Nevertheless, it must be pointed out that the application of structure-specific detection systems such as MS or Fourier transform infrared (FT-IR) spectroscopy does not eliminate the fundamental requirement to optimize (chiral) resolution and maximize effective sample clean-up.

Gas Chromatography - Isotope Ratio Mass Spectrometry (GC-IRMS). Isotope ratio measurements were performed with a Finnigan MAT delta S isotope mass spectrometer, on-line coupled to a Varian 3400 GC via a combustion interface.

The isotope mass spectrometer was calibrated against CO_2 gas with a defined $^{13}C/^{12}C$ content relative to the "PDB"-standard (Pee Dee Belemnite, fossil $CaCO_3$, yielding CO_2 of definite isotopic ratio $^{13}C/^{12}C = 0.0112372$) [4]. Further details concerning instrumentation and chromatographic conditions are reported elsewhere [1, 5, 6].

Results and Discussion

Enantio-MDGC. Enzymatic reactions are commonly characterized by a high degree of stereospecificity. Against this background naturally occurring flavor compounds of high enantiomeric purity should be expected. Thus in order to

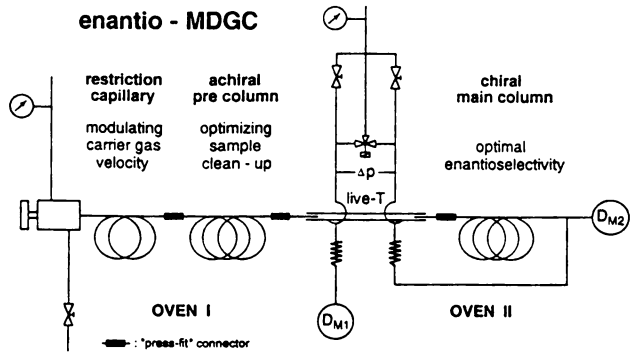

Fig. 1
Schematic diagram of enantio-MDGC according to ref. 2.

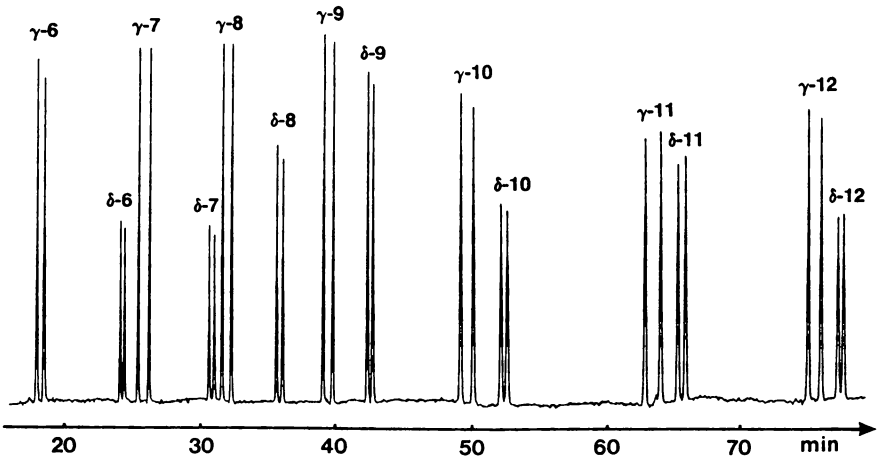

Fig. 2
Enantio-MDGC analysis of c(d)-lactones (standard mixture, main column separation); order of elution: c-C6-C12, δ-C6, δ-C7 R(I), S(II); δ-C8-C12 S(I), R(II), according to refs. 8, 9.

obtain accurate information with respect to chirality, analytical procedures of highest selectivity, which employ chiral separation without racemization must be utilized. In addition, references of definite chirality are essential in chirospecific analysis.

Three types of limitations have to be accepted: racemates of natural origin, generated by non-enzymatic reactions (autoxidation, photooxidation, etc.); racemization during processing or storage of foodstuffs, if structural features of chiral compounds are sensitive; and blending of natural and synthetic chiral flavor compounds. Nevertheless, the systematic evaluation of origin-specific enantiomeric ratios has proved to be a valuable criterion for differentiating natural flavor compounds from those of synthetic origin.

γ(δ) Lactones. While the direct analysis of single sets of chiral compounds and their homologues was reported in the early accounts of enantioselective flavor analysis (**2**), simultaneous stereodifferentiation of chiral compounds with different functionalities has subsequently been achieved [7]. Using enantio-MDGC the simultaneous stereo-analysis of γ(δ)-lactone has been recently reported (Figure 2) [**8, 9**]. This analysis was applied to raspberries proving that the (S)-configurated enantiopure δ-octa- and δ-decalactone can be used as indicators of genuineness during all stages of fruit processing (Figure 3). In contrast, aroma relevant amounts of γ-lactones from raspberries were not detectable.

Even the higher homologues of δ-lactones (C13-C18) could be resolved into their enantiomers, using a thin film capillary coated with modified ß-cyclodextrin [10], fig. 4.

2-Methylbutanoic acids (esters). 2-Methylbutanoic acid and its esters have been identified as enantiopure, (S)-configured compounds. They have been found in natural sources like apples, pineapples, strawberries and many other fruits [7]. Enantiopure (S)-2-methylbutan-1-ol could also be detected from fermented food products (alcoholic beverages). (S)-2-methylbutan-1-ol is well established as the starting material of natural 2-methylbutanoic acid (esters) by biotechnological procedures, yielding (S)-enantiomers as well. As shown in Figure 5, all the homologues of 2-methylbutanoic acid esters as well as 2-methylbutylacetate can be separated into their enantiomers. Thus, synthetic racemates can be easily detected from their natural analogues [11].

Buchu leaf oil. Because of its powerful, typical "cassis" flavor note, buchu leaf oil, the steam distilled oil of *Agathosma betulina* Barth. or *Agathosma crenulata* (L.) Hook, is frequently used as a valuable flavor ingredient in fruit aromas and fragrance compositions. During our investigations on structure-function relationship of buchu leaf oil`s flavor impact compounds, the stereoisomers of 3-oxo-p-menthane-8-thiol (**9-12**) and their thiol acetates (**13-16**) have been chirospecifically analyzed and their main sensory properties have been described. As shown in Fig. 6, the use of enantio-MDGC allows the most interesting chiral compounds of buchu leaf oil to be separated into their mirror image components in just one chromatographic run. Figure 7 shows an actual buchu leaf oil analysis. The upper chromatogram shows the resultant enantio-MDGC separation, whereas

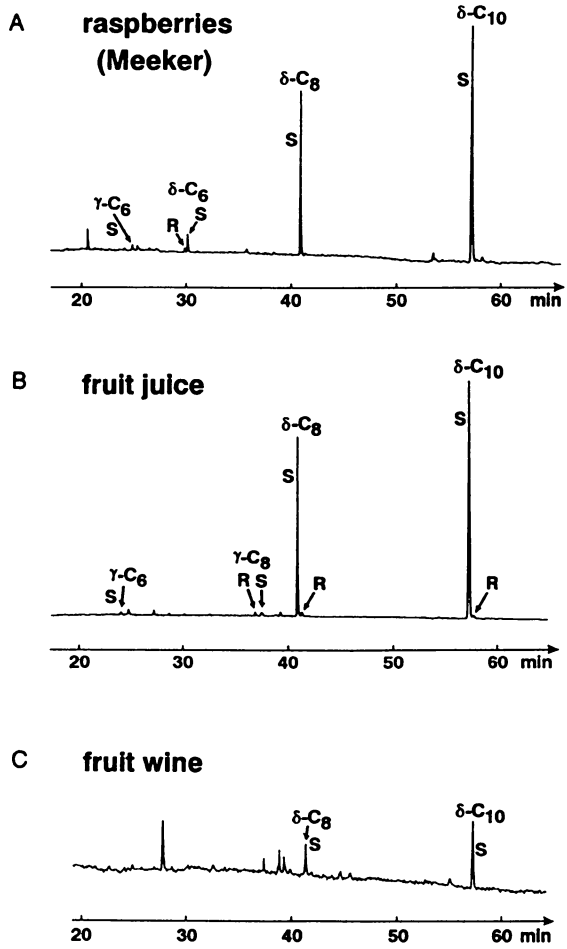

Fig. 3
Stereoanalysis (d)-lactones from raspberries; A: fresh fruits; B: fruit juice, C: fruit wine from the same fruit sample, according to refs. 8, 9.

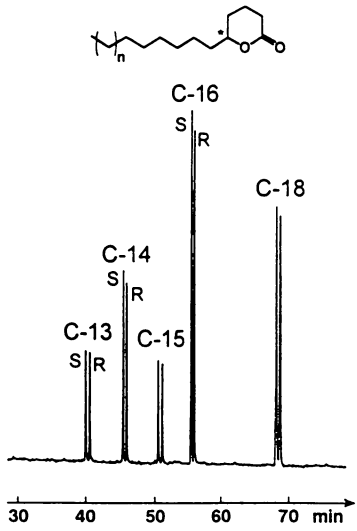

Fig. 4
Stereodifferentiation of higher homologous δ-lactones, C13-C18; from ref. 10.

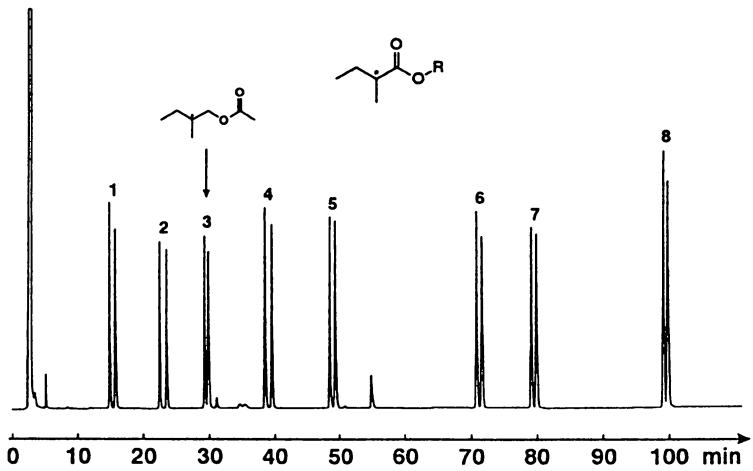

Fig. 5
Chiral separation of homologous 2-methylbutanoic acid esters: methyl-(1), ethyl-(2), propyl-(4), isobutyl-(5), isopentyl-(6), pentyl-(7) and hexyl-(8)esters; 2-methylbutylacetate (3); from ref. 11.

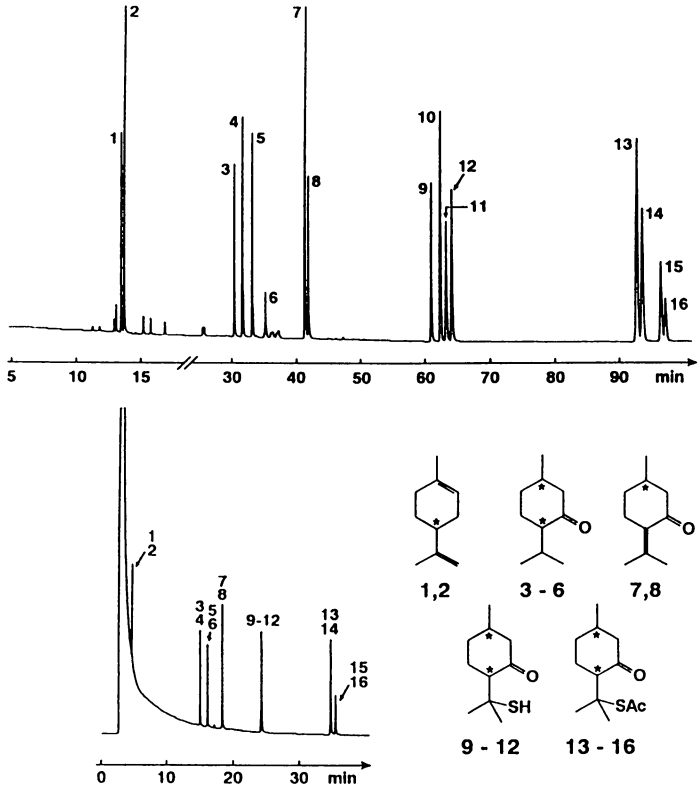

Fig. 6
Simultaneous stereodifferentiation of buchu leaf oil constituents, using enantio-MDGC analysis (standard mixture).
limonene: 1 (S), 2 (R); menthone: 3 (1S, 4R), 4 (1R, 4S); isomenthone: 5 (1R, 4R), 6 (1S,4S); pulegone: 7 (R), 8 (S); cis-3-oxo-p-menthane-8-thiol: 9 (1S, 4R), 11 (1R, 4S); trans-3-oxo-p-menthane-8-thiol: 10 (1R, 4R), 12 (1S, 4S); trans-3-oxo-p-menthane-8-thiol acetate: 13 (1R, 4R), 14 (1S, 4S); cis-3-oxo-p-menthane-8-thiol acetate: 15 (1S, 4R), 16 (1R, 4S). Preseparation of racemic compounds (A); chiral resolution of enantiomeric pairs (B); from ref. 12.

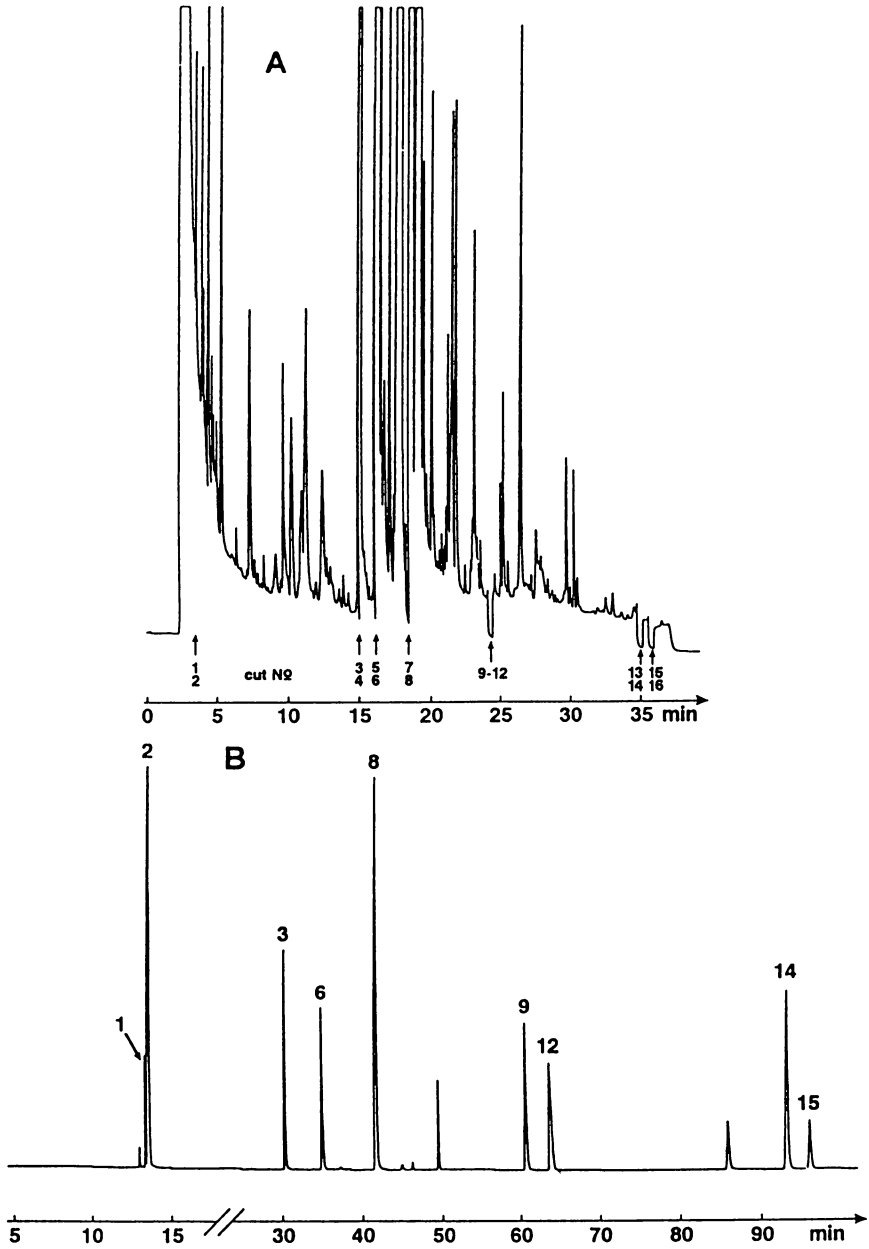

Fig. 7
Simultaneous enantio-MDGC of buchu leaf oil, self-prepared. A: pre column separation; B: main column separation, detecting (1S)-configurated compounds 3, 6, 8, 9, 12, 14, 15, according to ref. 12.

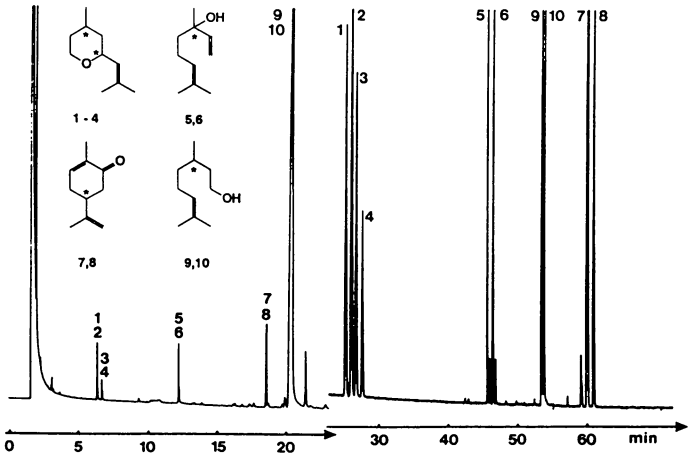

Fig. 8
Enantio-MDGC analysis of cis-rose oxides: 1 (2R, 4S), 2 (2S, 4R); trans-rose oxides: 3 (2R, 4R), 4 (2S, 4S); linalol; 5 (R), 6 (S); carvone: 7 (R), 8 (S); citronellol: 9 (S), 10 (R), Pre-separation of racemic compounds (left); chiral resolution of enantiomeric pairs (right); from ref. 13.

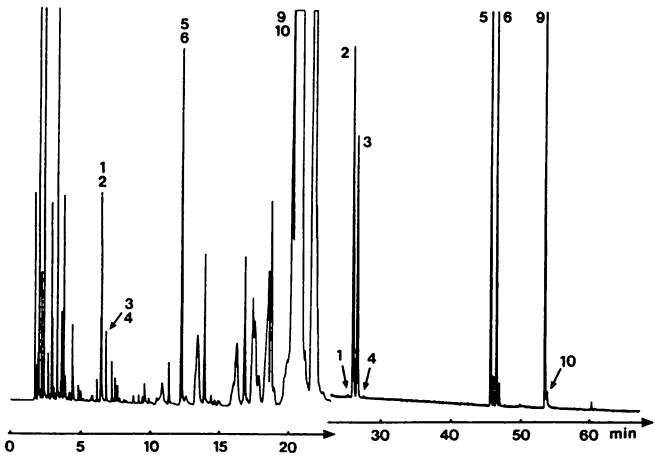

Fig. 9
Enantio-MDGC analysis of an authentic rose oils from Rosa damascena Miller (Turkey), detecting cis(-)-2, trans(-)-3 rose oxides and (S)(-)-citronellol (9) as genuine rose oil compounds; from ref. 13

the lower chromatogram shows the matrix from which the entiomertic cuts were made from. It should be noted that menthone (**3**), isomenthone (**6**), pulegone (**8**) the thiols (**9, 12**) and their thiolacetates (**14, 15**) were detected as enantiopure genuine constituents of buchu leaf oil [12].

Rose oils. Rose oils are always among the most expensive and precious essential oils. They are highly appreciated, particularly in fine perfumery. Using 6-O-silylated ß(γ)-cyclodextrins as a new generation of chiral stationary phases, the chiral monoterpenoids from rose oil were stereoanalyzed by enantio-MDGC. Shown in Figure 8 is the simultaneous chirality evaluation of cis-rose oxide (**1, 2**), trans-rose oxide (**3, 4**), linalol (**5, 6**), carvone (**7, 8**) as well as citronellol (**9, 10**) was achieved. Their order of elution was assigned by co-injection with optically active references of definite chirality .

Shown in Figure 9 are Chromatograms of certificated rose oils from Bulgaria or Turkey. The levorotatory (4R)-configured cis (**2**)- and trans (**3**)-diastereomers of rose oxides were detected as the genuine constituents with high enantiomeric purity (> 99.5 %). The authenticity of these samples is in full accordance with the enantiomeric purity (> 99 %) of (S)(-) observed for citronellol (**9**), a main compound in these oils. These findings suggest **9** as the biogenetic precursor of the genuine cis (**2**)- and trans (**3**)-rose oxides in authentic rose oils. Surprisingly, linalol (**5, 6**) was detected as a racemate. Meanwhile we have proved, that the chiral stability of linalol is affected by the technological conditions applied in rose oil production. As shown in Figure 10, detection of the unnatural diastereomers (+) cis-1 and (+)-trans-**4** rose oxides in commercially available rose oil samples is unambiguous proof of adulteration. Also the enantiomeric distribution of linalol (**5, 6**) and citronellol (**9, 10**) in this sample differ significantly from those of authentic rose oil [13].

The dextrorotatory compounds **1, 4** and **10** are identified as unnatural enantiomers, either being (partially) synthesized products or coming from foreign natural sources. In this context it is interesting to note the "sniffing" GC-olfactory analysis of rose oxide (**1-4**), linalol (**5, 6**) and citronellol (**7, 8**) enantiomers shown in Figure 11. The natural cis (**2**)-rose oxide exhibits a powerful rosy scent and about 0.4 ng can be sniffed, while the other stereoisomers **1, 3, 4** even about 300 ng could not sensorially be recognized. (R)(-) linalol (**5**) shows a flowery, pleasant scent. The scent of the (S)(+) linalol (**6**) is similar to that of (R)(-) **5**, but less intensive. Finally (S)(-) citronellol (**7**) exhibits a sweet, flowery, rosy scent; the scent of (R)(+) (**8**) is similar to that of the (S)(-) **7** enantiomer and in the same odor activity range [14].

Dihydrofuranones. Chiral 3[2H] and 2[5H] furanones are known to be important flavor compounds. The first chirospecific cGC analysis of 2,5-dimethyl-4-hydroxy--3-[2H]-furanone and the corresponding methylether, using permethyl-ß-cyclodextrin as the chiral stationary phase has already been reported [15]. The systematic development of modified cyclodextrin phases has allowed new possibilities in enantiomer separation. 4,5-Dimethyl-3-hydroxy-2[5H]-furanone (**9**), the so-called "sotolon", is a well known flavor compound, imparting intense spicy

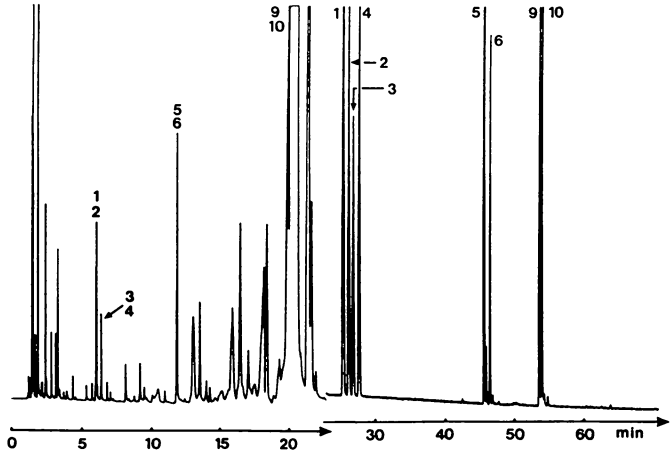

Fig. 10
Commerically available rose oil, adulterated by the addition of unnatural enantiomers cis(+)-1, trans(+)-4 rose oxides and (R)(+)-citronellol (10); from ref. 13.

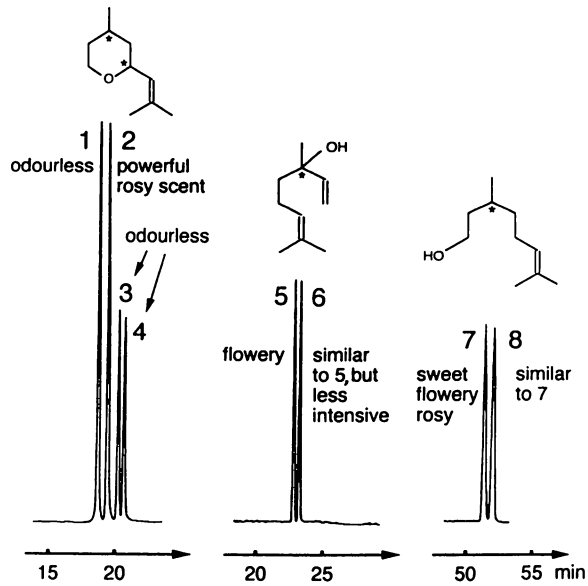

Fig. 11
Enantioselective cGC separation and odor impression by enantiomer eluate sniffing of cis rose oxides: 1 (2R, 4S), 2 (2S, 4R) and trans rose oxides: 3 (2R, 4R), 4 (2S, 4S); from ref. 14.

tastes and odors. The chiral separations of it and its methyl ether are shown in Figure 12. Because of their high odor activities, 2[5H]-furanones are interesting molecules for studying the relationship between chirality and odor impression, presupposing isomers of high enantiomeric excess. As observed in Fig. 12 chirospecific analysis with 6-O-silylated cyclodextrins revealed extreme separation factors, in particular for the free enolic structure (**9**).

The odor impression of (5R)-**9** and (5S)-**9** are rather similar, however considerable differences in odor thresholds exist. On the other hand the odor activity of the corresponding methylether (**13**) decreased significantly in the case of (5S)-13 or disappeared completely in the case of (5R)-**13**. The order of elution is also inverted. Natural "sotolon" from fenugreek seed was identified as favoring the (5S)-**9** enantiomer (92.6 % ee) [16, 17].

GC-IRMS. The elucidation of stable isotope distributions is highly desirable with respect to fundamental studies in biochemistry, nutrition, drug research and also in the authenticity control of flavors and fragrances. Capillary gas chromatography, cGC, coupled on-line via a combustion interface with isotope ratio mass spectrometry, IRMS, has been employed successfully in the case of $\delta^{13}C$ determinations. The substances eluting from the cGC column are converted into carbon dioxide in a combustion oven and then directly analyzed in the isotope mass spectrometer. The spectrometer is adjusted for the simultaneous recording of the masses 44($^{12}C^{16}O_2$), 45($^{13}C^{16}O_2$, $^{12}C^{16}O^{17}O$) and 46($^{12}C^{16}O^{18}O$). These components can be detected in the nmole range and with high precision < 0.3 [o/oo] [18-20].

The isotope ratio traces of the GC peaks exhibit a typical S-shape. The heavier isotopic species of a compound elute more rapidly from the cGC-column than the light species. Similar effects can be observed for all chromatographic processes, whereas the size of isotope fractionation and the elution-order of the isotopomers depends on: 1.) the chromatographic system applied, 2.) the temperature, and 3.) the structural features of compounds analyzed. In any case, care must be taken to integrate across the full width of the chromatographic peaks. Of course, reliable results on isotopic ratios from cGC-IRMS experiments can only be expected from very high-resolution cGC (Rs > 1.5). Also, accurate sample clean-up procedures without any isotope fractionation must be guaranteed.

The importance of quantification of chromatographic processes is demonstrated with a sample of decanal [5]. Shown in Figure 13, section A, is the quantitative separation -29.42 [o/oo] of the HPLC fractionation. In section B fractions F2: -34.37 [o/oo] and F3: -24.60 [o/oo] are shown. The fraction F2 and F3 of this peak are significantly different ($\Delta\delta^{13}C$: 9.77 [o/oo]). Obviously the heavier isotopomers are more retarded (F3), whereas the lighter species are eluted first (F2). As shown in section C that heart cutting also yields different $\delta^{13}C$ values, i.e., F4: -28.55 [o/oo] and F5 + F6: 30,04 [o/oo].

Under the conditions of: accurate sample clean-up, high resolution (baseline: Rs > 1.5) and quantitative peak evaluation, HPLC, HPTLC, PHSC (Preperative High Resolution Segment Chromatography) chromatographic procedures have proven to be reliable methods in concentrating natural flavor compounds from complex matrices. Thus, GC-IRMS may be used as a suitable tool in evaluating precise δ^{13}CPDB-measurements [21].

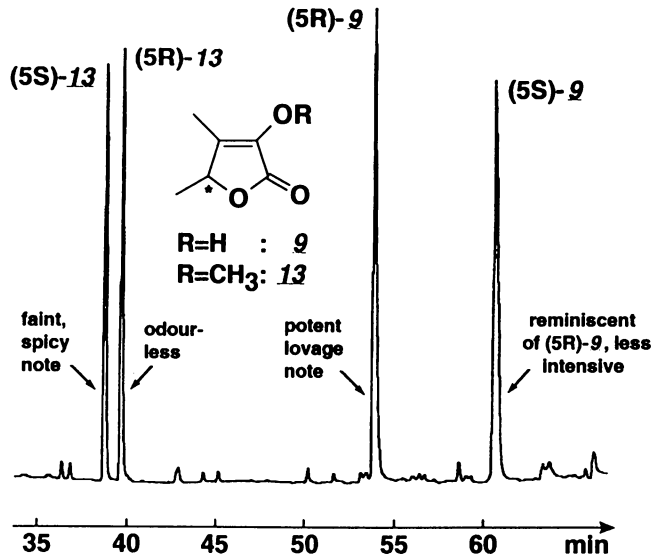

Fig. 12
Enantio selective cGC separation and odor impression by enantiomer eluate sniffing of 2[5H]-furanones (9, 13); from ref. 16, 17.

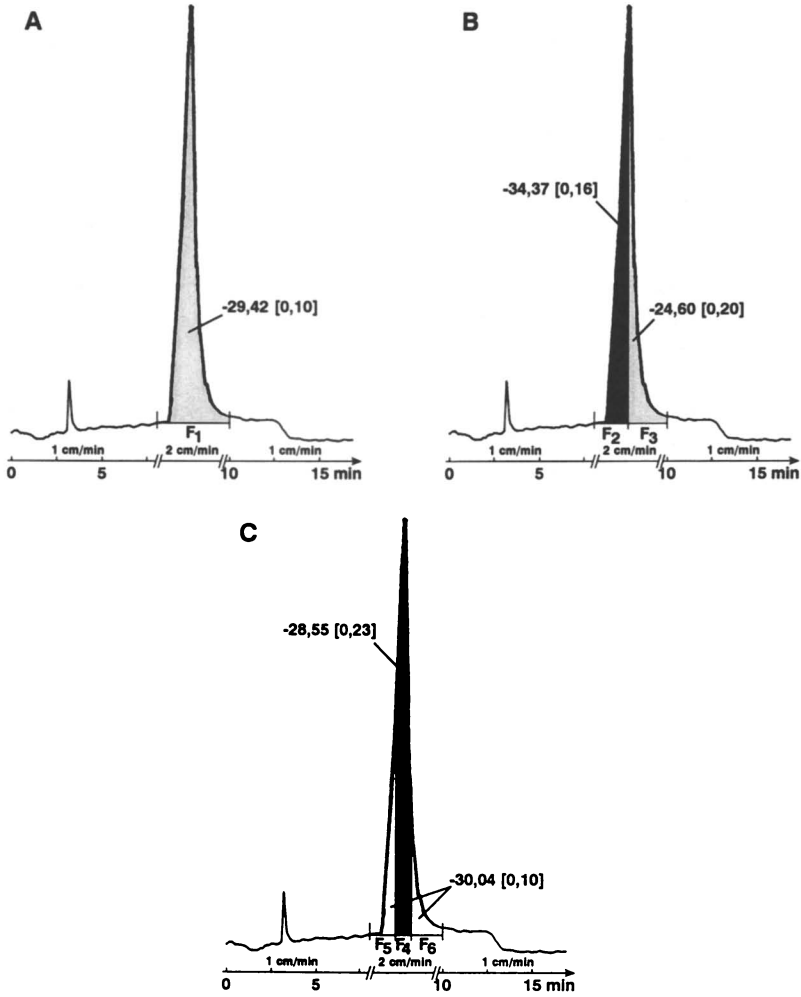

Fig. 13
Isotope discrimination of decanal by prep. HPLC δ13CPDB-values [o/oo], according to ref. (5). Section A is the quantitative separation -29.42 [o/oo] of the HPLC fractionation. Section B fractions F2: -34.37 [o/oo] and F3: -24.60 [o/oo] are shown. Section C shows that heart cutting also yields different $\delta^{13}C$ values, i.e., F4: -28.55 [o/oo] and F5 + F6: 30,04 [o/oo].

But unfortunately, the usability of IRMS-analysis in the authenticity control of flavor compounds is limited, as most plants cultivated for human nutrition belong to the group of C3-plants yielding δ^{13}CPDB-values partially over-lapping with those of synthetic substances from fossil sources. To overcome this principal limitation we propose the definition of suitable internal isotopic standards (i-IST). In this way, the main influence of isotope discrimination, caused by CO_2 fixation during photosynthesis is eliminated. Isotopic effects among genuine compounds are, therefore, limited to the influence of enzymatic reactions during secondary biogenetic pathways [6].

The fingerprint of lemon oil compounds is shown in fig. 14: where ß-pinene (**1**), limonene (**2**), c-terpinene (**3**), nerol (**4**), geraniol (**5**), neral (**6**), geranial (**7**), nerylacetate (**8**), geranylacetate (**9**) based on δ^{13}C-values [o/oo], are calculated versus nerylacetate (**8**). Calculating nerylacetate (**8**) as an internal versus an i-IST yields fruit specific δ^{13}C-values for neral (**6**) and geranial (**7**) of 5 lemon oils (different provenance) and with low standard deviation. That means $\delta^{13}C_{PDB}$ level of compound (**8**) is set as the new zero point. Now all the other monoterpenoids are measured relative to this *i-IST* compound (**8**). This integrated evaluation is a new and promising way in the authenticity control of flavors and fragrances as well as in studies on biochemical pathways.

In order to choose a suitable i-IST, the following principal aspects have to be taken into account:

1. The substance, selected as an i-IST should be a characteristic genuine compound of less importance in view of sensorial relevance.
2. The compound must be available in sufficient amounts and free of isotopic discrimination during sample clean-up.
3. The selected compound should be biogenetically related to the compounds under investigation.
4. Its chemical inertness during storage and/or technical processes is mandatory.

Finally we have introduced the system enantio-cGC combined with IRMS and have called it enantio-IRMS [22-24]. Enantio-IRMS detects enantiomers of the same source as enantiomeric ratios with identical δ^{13}C-levels. As outlined in fig. 15, linalol from coriander oil is detected as an R(20 %) : S (80 %) enantiomeric ratio with identical isotope levels of the enantiomers.

Enantio-IRMS also offers a direct method to detect conclusively a blend of enantiopure chiral flavor compound with synthetic racemate. An origin specific enantiomeric ratio may be imitated, but is not yet detectable, neither by chirospecific analysis nor by IRMS-measurements. However, in the case of enantio-IRMS a simulated origin-specific enantiomeric distribution is proved by different δ^{13}C levels of the detected enantiomers (Fig. 16). An earlier investigation proved, that up to 8 % of (S)(+) linalol may be generated by hydrodistillation (2 h) of lavender [25]. Although hydrodistillation doesn't correspond with GMP-conditions of lavender oil production, we propose to accept 15 % of (S)(+) linalol as the maximum from technologically induced partial racemization of genuine (R)(-) linalol from lavender. In fig. 16 the experiments to reveal the origin of linalol from a commercially available spike oil are compared

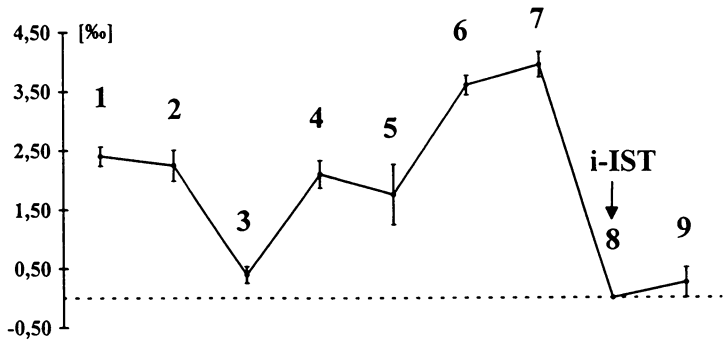

Fig. 14
Fingerprint of lemon oil compounds, based on δ13C values [o/oo], calculated versus nerylacetate (8), according to ref. (6).

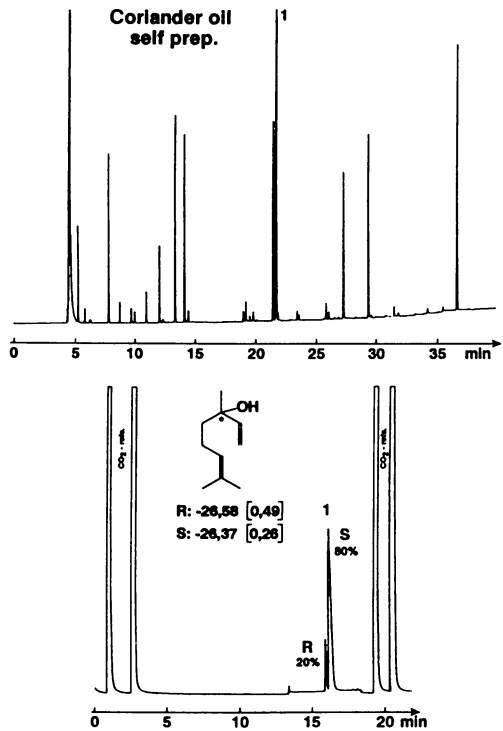

Fig. 15
Enantio-IRMS analysis of linalol from coriander seed oil, self prepared; from ref. 26.

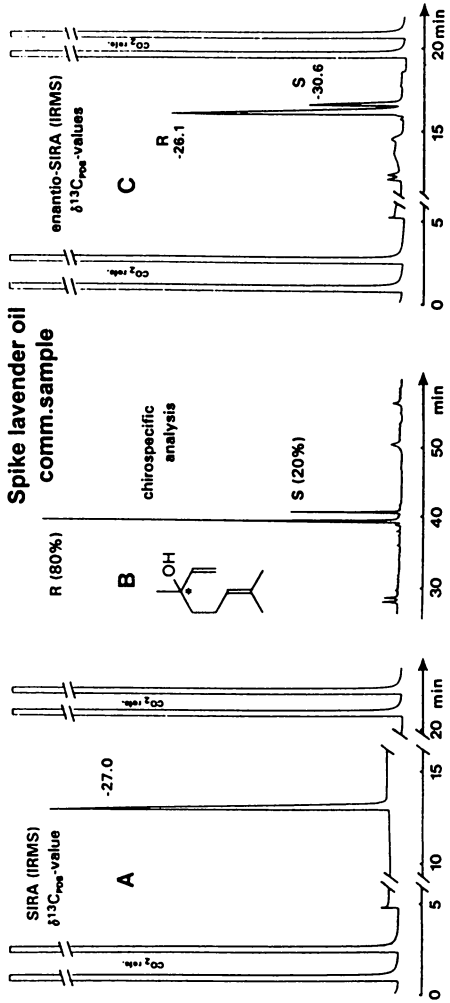

Fig. 16
cGC-IRMS (A), chirospecific analysis (B) and enantio-IRMS measurements (C) of linalol from a commercially available spike oil. δ13CPDB values [o/oo]: linalol -27.0 (+ 0.2); R-enantiomer -26.1 (+ 0.2); S-enantiomer -30.6 (+ 0.6); from ref. 23.

[23]. While chirospecific analysis detects the R(80%) : S(20%) enantiomeric ratio, indicating a blend with synthetic racemate, the amount of synthetic racemate cannot be calculated, owing to a conceivable partial racemization of linalol during the processing of Lavandula oils. By means of enantio-IRMS investigation the blend of linalol from different origin is proved [R(-26.1 o/oo), S(-30.6 o/oo)], whereas simple IRMS-analysis [R+S (-27.0 o/oo)] even simulates the occurrence of a genuine compound, due to the overlapping ranges of δ^{13}C-values of substances from C3-plants with those prepared from fossil sources.

Acknowledgments

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Chapter 10

Starfruit (*Averrhoa carambola* L.)

Attractive Source for Carotenoid-Derived Flavor Compounds

Peter Winterhalter, Andrea Lutz, Markus Herderich, and Peter Schreier

Lehrstuhl für Lebensmittelchemie der Universität Würzburg, Am Hubland, 97074 Würzburg, Germany

Carotenoid derived compounds are important contributors to the overall flavor of starfruit. Recent research has shown that the majority of norisoprenoid volatiles in starfruit are derived from glycosidically bound progenitors. This chapter reports the isolation and structural elucidation of several novel C_{13}- and C_{15}-norisoprenoid structures in starfruit. The co-occurrence of these C_{13}- and C_{15}-carotenoid metabolites with volatiles which are apparently derived from the central portion of the carotenoid chain leads to a discussion of possible pathways of carotenoid breakdown in starfruit.

The aroma of starfruit (*synonym*: carambola) has been extensively studied and approximately 200 aroma substances have been identified to date (*1-6*). However, there are major differences in the aroma composition reported, especially with regard to the occurrence of C_{13}-norisoprenoid flavor compounds. Whereas in previous studies ß-ionone and ß-ionol were detected as sole C_{13}-norisoprenoids (*3,4*), recent findings by MacLeod and Ames (*5,6*) indicated the presence of a large number of structurally related C_{13}-compounds in starfruit. This discrepancy in the aroma composition is apparently due to the different isolation procedures used, since in the latter case simultaneous distillation/extraction (SDE) (*7*) at atmospheric pressure has been applied - a technique which is known to generate "secondary" volatiles by degradation of acid-labile precursors.

Identification of glycosidically bound norisoprenoid flavor precursors in starfruit

To gain information about the presence of such aroma precursors in starfruit, glycosidic isolates were prepared by XAD-2 adsorption (*8*) and subsequent methanol elution. This glycosidic extract was enzymatically hydrolyzed with sweet almond emulsin. C_{13}-aglycons liberated by this treatment are shown in Fig. 1 (*9*). Among these apparently carotenoid-derived metabolites several important aroma precursors can be found, including a rare natural precursor of the intensely odorous ß-damascenone, i.e. allenic triol **14** (*10,11*). Further reactive precursors are 4-hydroxy-ß-ionol **1**, 3-hydroxy-ß-ionol **2**, 3-hydroxy-ß-ionone **3**, 3-oxo-α-ionol **5**, and 3-hydroxy-5,6-epoxy-ß-ionol **16**. Their specific role as aroma precursor has been discussed elsewhere (*9,12,13*).

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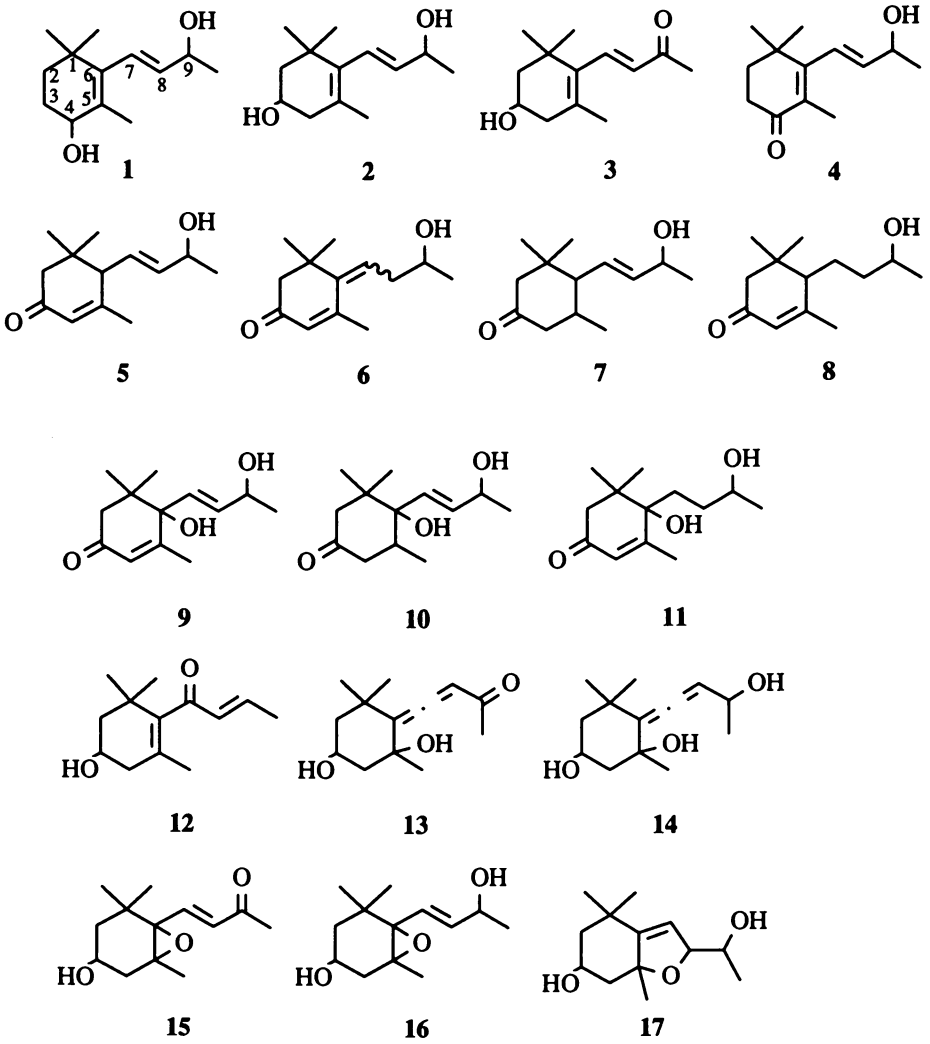


Figure 1. Structures of enzymatically-liberated C_{13}-norisoprenoid aglycons **1-17**: 4-hydroxy-ß-ionol **1**, 3-hydroxy-ß-ionol **2**, 3-hydroxy-ß-ionone **3**, 4-oxo-ß-ionol **4**, 3-oxo-α-ionol **5**, 3-oxo-*retro*-α-ionol **6**, 3-oxo-4,5-dihydro-α-ionol **7**, 3-oxo-7,8-dihydro-α-ionol (Blumenol C) **8**, vomifoliol **9**, 4,5-dihydrovomifoliol **10**, 7,8-dihydrovomifoliol **11**, 3-hydroxy-ß-damascone **12**, grasshopper ketone **13**, megastigma-6,7-diene-3,5,9-triol **14**, 3-hydroxy-5,6-epoxy-ß-ionone **15**, 3-hydroxy-5,6-epoxy-ß-ionol **16**, and 3-hydroxy-actinidol **17**.

MLCCC isolation of a new C_{13}-norisoprenoid glycoside from starfruit

In addition to the norterpenoid aglycons shown in Fig. 1, mass spectral data indicated the occurrence of several unknown C_{13}-aglycons in starfruit. In an effort to elucidate their structure, an isolation of the intact glycoconjugates was attempted. For the separation of starfruit glycosides, the all-liquid chromatographic technique of multi-layer coil countercurrent chromatography (MLCCC) was applied (*14*). This technique possesses several advantages with regard to the analysis of polar natural products, such as glycosides: (i) MLCCC as a liquid-liquid chromatographic technique does not employ solid adsorbents, i.e. there are no adsorption losses and thus the formation of artifacts is minimized. (ii) Compared with other CCC-techniques, such as Droplet-CCC (*15,16*) or Rotation Locular-CCC (*17,18*), MLCCC is reported to possess a considerably enhanced separation power. (iii) Utilizing large diameter coils, MLCCC enables preparative separations in a short time. Fig. 2 summarizes commercially available CCC-systems, all of them being routinely used in natural product analysis.

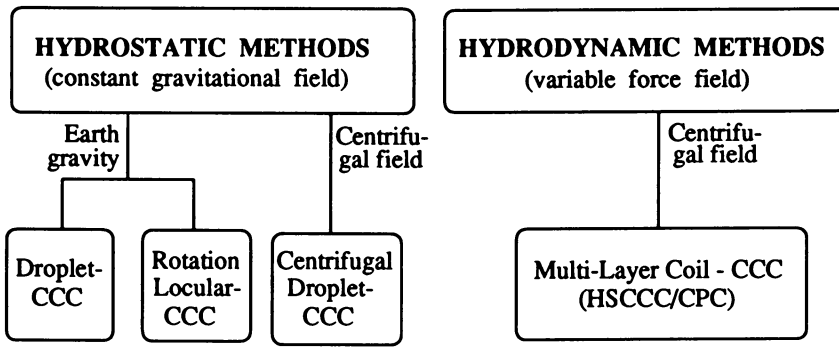


Figure 2. Commercially available CCC-equipment, i.e. low-resolution 'hydrostatic' techniques of *droplet countercurrent chromatography* (DCCC), *rotation locular countercurrent chromatography* (RLCCC), and *centrifugal droplet countercurrent chromatography* (CDCCC) as well as high-resolution 'hydrodynamic' techniques of *multi-layer coil countercurrent chromatography* (MLCCC) [synonym: *high speed countercurrent chromatography* (HSCCC) or *centrifugal partition chromatography* (CPC)].

In the present study separations have been carried out with a Multi-Layer Coil Separator-Extractor (P.C. Inc., Potomac). This apparatus uses a Teflon tube, which is wrapped around a holder in several layers, as the separating column. This so-called "multi-layer coil" contains a biphasic solvent mixture, which - due to the rotation of the coil in a planetary system - is exposed to quickly changing centrifugal field forces, thus providing a highly efficient partitioning of the solutes. The apparatus has originally been designed by Ito and coworkers (*14*), detailed descriptions of the technique as well as numerous applications can be found in the literature (*19-23*).

For the isolation and purification of glycoconjugates from complex natural matrices a two-step procedure has been developed recently (*24,25*). The two-step procedure consists of a large-scale preseparation of the glycosidic mixture on a

"preparative coil" (i.e., a 75 m x 2.6 mm i.d. PTFE tubing) and a further purification of preseparated fractions on an "analytical coil" (160 m x 1.6 mm i.d. PTFE tubing) using optimized solvent systems. Whereas major glycosides are generally obtained after MLCCC in sufficient purity, thus allowing immediate structural elucidation by NMR, less abundant glycoconjugates are finally purified by semipreparative and/or analytical HPLC.

Also for the unknown starfruit glycosides a combination of MLCCC and HPLC purification steps had to be carried out. From 24 kg of the fruit the target glycosides were isolated in pure form and characterized as their peracetates (*26*). Peracetylated glycoside **18** (2.5 mg) had a molecular mass of 600 (C_{29} $H_{44}O_{13}$). From the ^{1}H- and ^{13}C-NMR data the unknown compound was identified as the ß-D-glucopyranoside of 3,6-dihydroxy-5,6-dihydro-ß-ionol **18** (cf. Fig. 3). This glucoside is reported for the first time in nature. For glycoside **19** (3.5 mg) a molecular mass of 556 ($C_{27}H_{40}O_{12}$) was determined. Its spectral data were in good agreement with data published for 3,6-dihydroxy-5,6-dihydro-ß-ionone 3-O-ß-D-glucopyranoside, a C_{13}-glucoside first isolated from leaves of the African medicinal plant *Boscia salicifolia* (*27*). Mass spectral data for the newly identified starfruit aglycons **14**, **18**A and **19**A are gathered in Fig. 4.

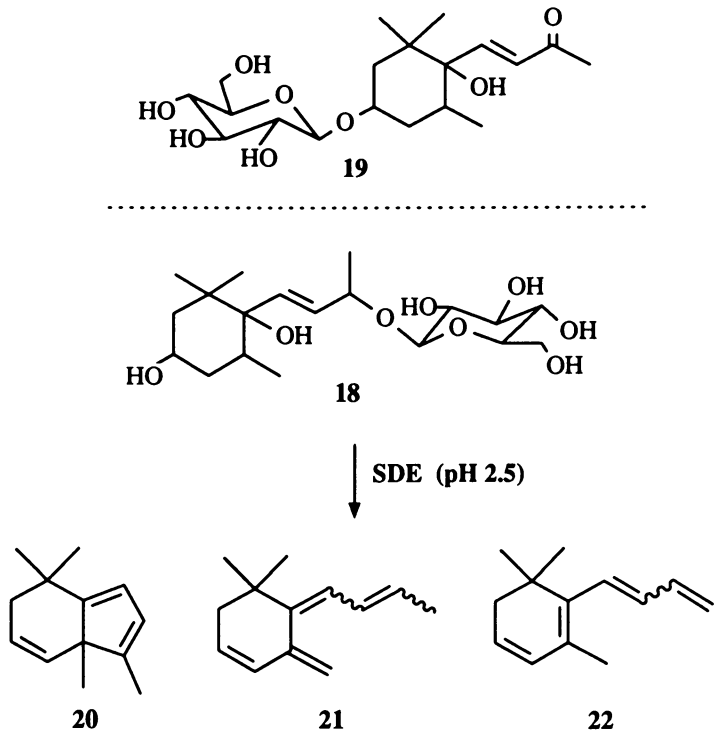


Figure 3. Structures of newly identified starfruit glucosides **18** and **19** as well as degradation products **20-22** obtained by SDE-treatment (pH 2.5) of precursor **18.**

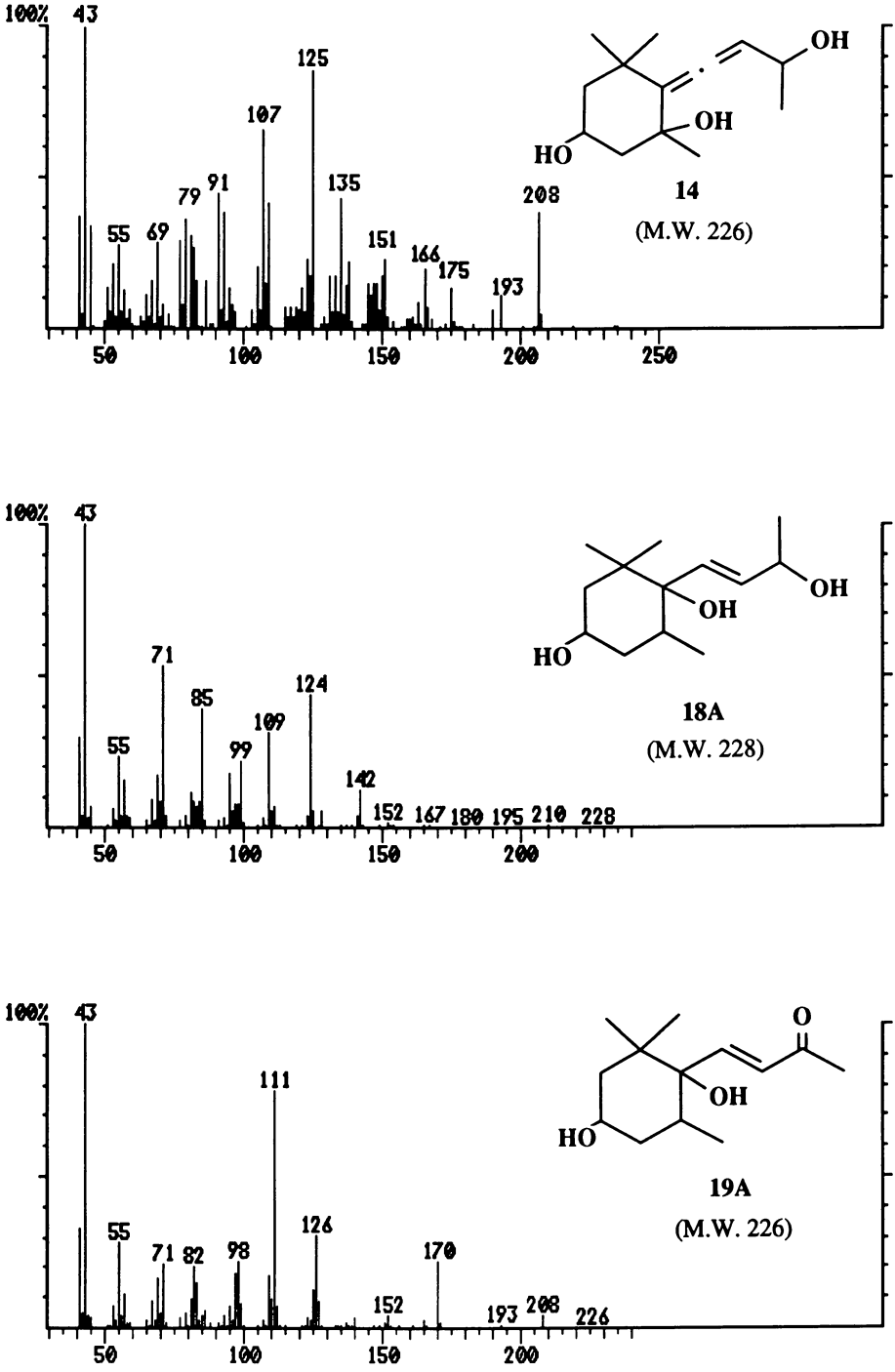


Figure 4. Mass spectral data (70 eV) of starfruit aglycons **14**, **18A**, and **19A**.

Model hydrolytic studies. Continuing our studies on C_{13}-norisoprenoid aroma precursors, the possible role of the newly identified starfruit glycosides **18** and **19** as aroma precursors was studied. Under SDE conditions (pH 2.5) **18** was completely degraded to a series of isomeric hydrocarbons, for which on the basis of MS and FTIR data the structures **20-22** (cf. Fig. 3) have been proposed (*12*).

Identification of C_{10}- and C_{15}-carotenoid breakdown products in starfruit

In addition to numerous C_{13}-norisoprenoid constituents, starfruit was also found to contain C_{10}- and C_{15}-carotenoid metabolites. Initially, only the isomeric marmelo lactones **23a/b** have been identified in starfruit aroma extracts isolated by SDE-treatment. Importantly, the generation of these isomeric lactones could recently been explained by cyclization of glycosidically bound 2,7-dimethyl-8-hydroxy-4(*E*),6(*E*)-octadienoic acid **24**, which was isolated by us from quince fruit (*28*). This finding allowed to suggest a cleavage of quince carotenoids as schematically outlined in Fig. 5 (*29-31*). Although the concentration of marmelo lactones **23a/b** in the present study was low, it was reasonable to assume that the remaining C_{15}-endgroup should also be detectable in starfruit.

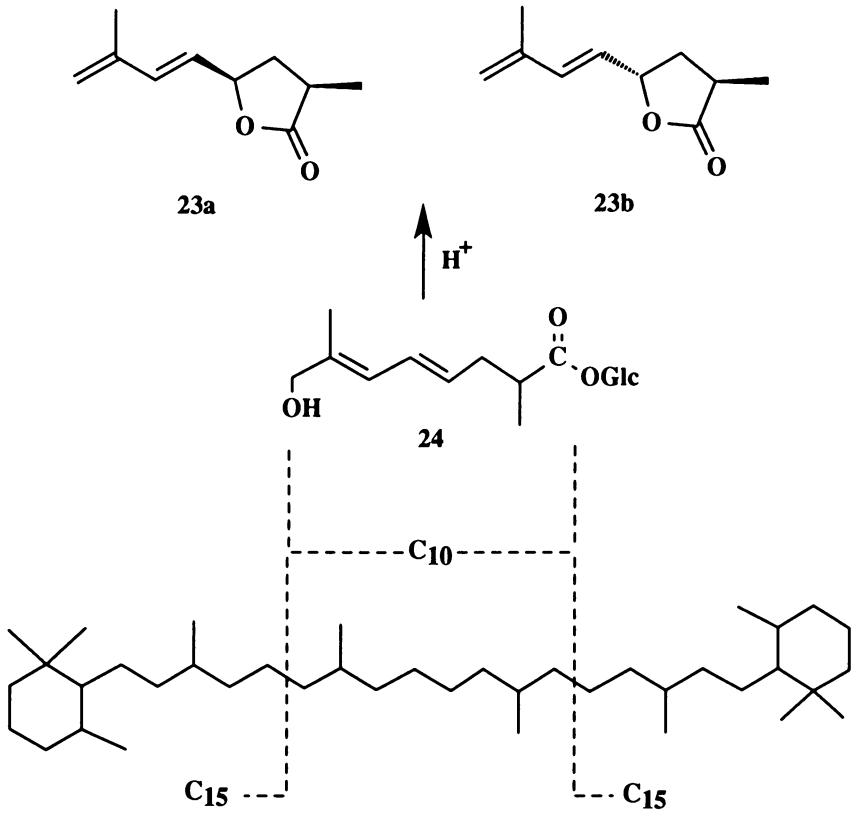


Figure 5. Postulated cleavage of carotenoids rationalizing the formation of C_{10}- and C_{15}-compounds.

Polar extracts of starfruit have therefore been prepared by the following methods:

(i) non-glycosidic constituents were continuously extracted with diethylether,
(ii) glycosidic constituents were obtained by XAD-2 adsorption and subsequent MeOH elution.

These extracts were screened for C_{15}-constituents using HRGC-MS (i) as well as enzymatic hydrolyses and HRGC-MS (ii), respectively. Free and bound C_{15} structures were purified by MLCCC and/or HPLC and characterized by NMR techniques. The structures of the newly identified C_{15}-constituents **25-28** in starfruit are shown in Fig. 6, their mass spectral data are gathered in Fig. 7.

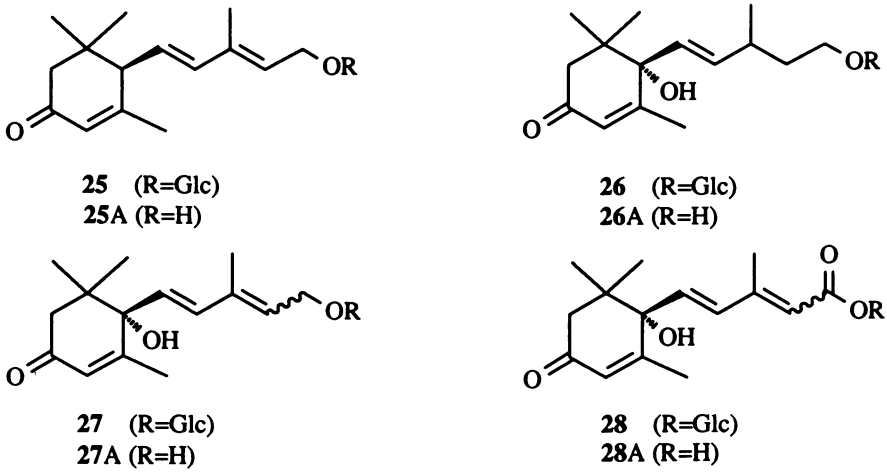
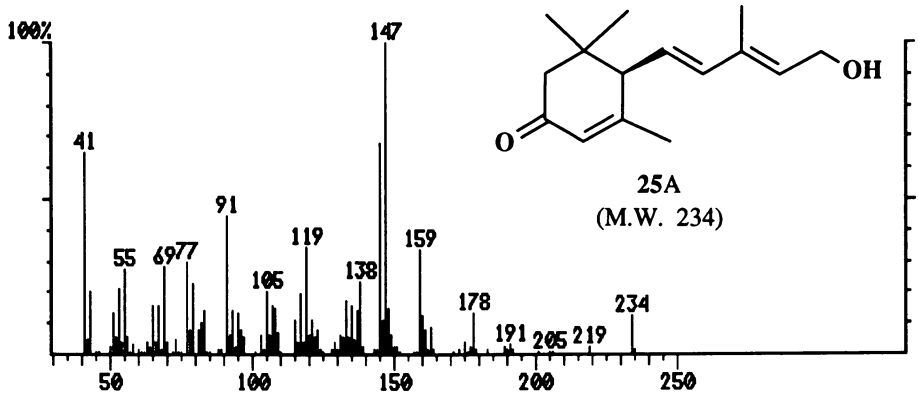
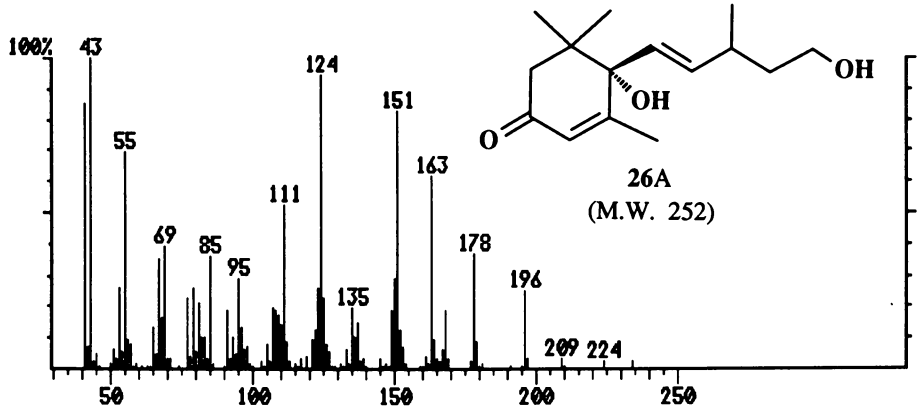


Figure 6. Structures of newly identified glycosidically bound C_{15}-compounds **25-28**. Aglycons **26A** and **27A** were furthermore present as free starfruit constituents.

Structurally, all these C_{15}-compounds are related to the important plant hormone abscisic acid (ABA) **28A** (*32*), which was also isolated as glucose ester **28** from the glycosidic isolate of starfruit. In the case of ABA much progress has been made recently in elucidating possible biosynthetic pathways. The evidence is now conclusive for the indirect "apo-carotenoid" pathway shown in Fig. 8, which proceeds by oxidative cleavage of epoxycarotenoids to xanthoxin. The latter is then metabolized to ABA *via* ABA-aldehyde. The oxidase activities that convert xanthoxin to ABA have been at least partially characterized (*33*). Alternatively, a shunt pathway from ABA-aldehyde *via* ABA-alcohol to ABA has been established which involves cytochrome P 450 monooxygenase conversion of ABA-alcohol to ABA (*34*).





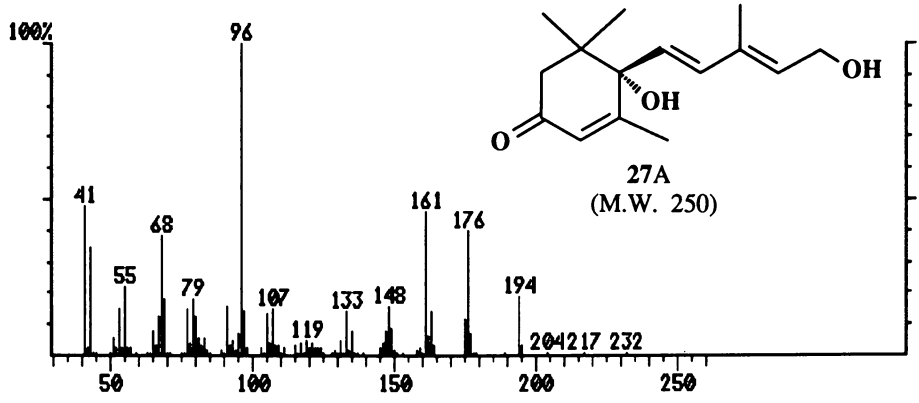


Figure 7. Mass spectral data (70 eV) of C_{15}-aglycons 25A-27A.

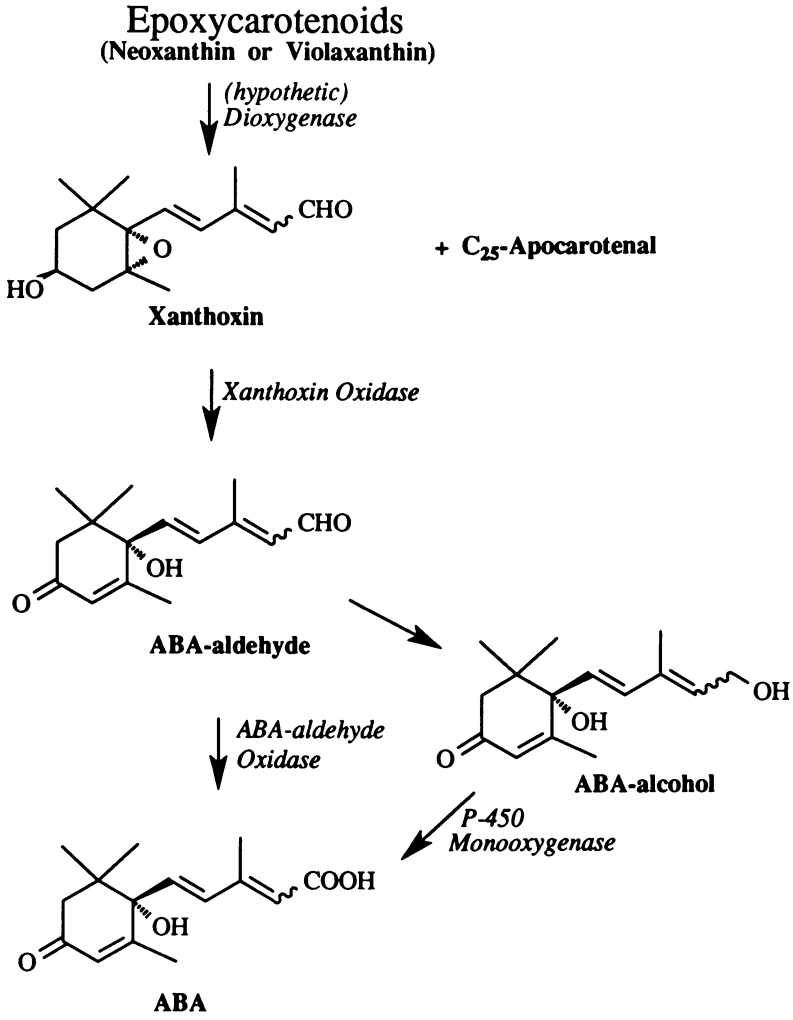


Figure 8. Proposed pathway for ABA formation (adapted from ref. *34*).

Quantitatively, C_{15}-structures predominate amongst the free starfruit volatiles (57% of total), and also constitute an important group of glycosidically bound constituents (32%). This abundance of C_{15}-constituents and the finding of numerous C_{13}-structures with a 3-oxo-group, i.e. aglycons **5-11**, prompted us to investigate possible enzymatic conversions of C_{15}- to C_{13}-compounds in starfruit. A first proposal for such a cleavage reaction has been made by Isoe in the early seventies (*35*). In our experiments ABA was therefore subjected to a "co-oxidation" reaction with lipoxygenase (LOX). In the presence of certain fatty acids, such as linoleic acid, LOX is known to cause carotenoid cleavage *in vitro* (*36-38*). Also in the case of ABA the known co-oxidative activity of LOX was observed, as can be seen from Fig. 9, in which dehydrovomifoliol **29** as C_{13}-degradation product could be detected.

From this key intermediate enzymatic pathways are expected to lead to further derivatives, including aroma precursors, such as, e.g., 7,8-dihydrovomifoliol **11**. Analogously, oxidative cleavage of the 6-desoxy ABA-derivative **25** theoretically allows access to 3-oxo-α-ionol **5** and derivatives **6-8** *via* 3-oxo-α-ionone.

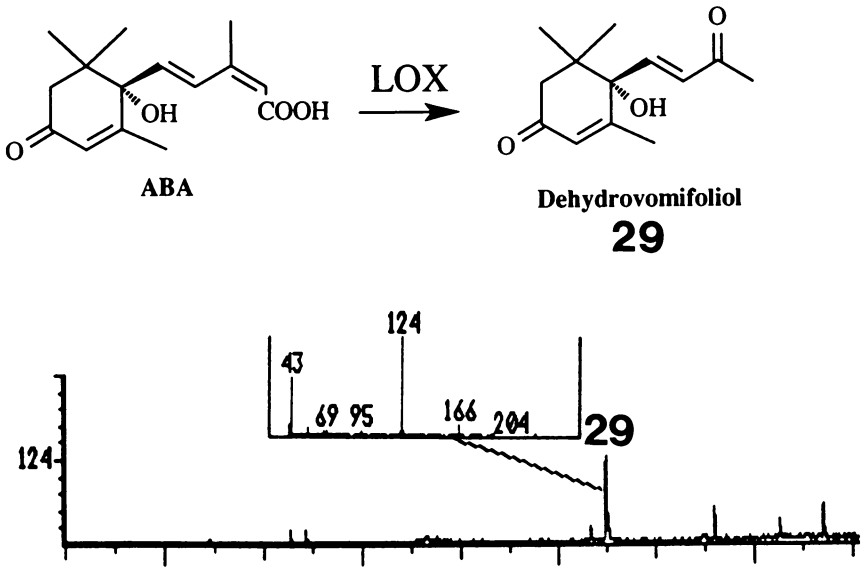
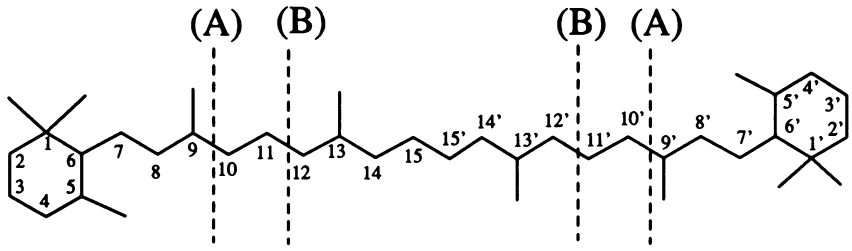


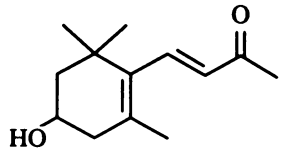
Figure 9. Co-oxidation of ABA revealing a formation of dehydrovomifoliol **29** as C_{13}-degradation product. *Experimental conditions:* (i) Co-oxidation: 10 mg LOX from soybean, linoleic acid (0.01 M) with 0.2% Tween 20, ABA (0.01 M) in phosphate buffer (0.1 M, pH 6.5); (ii) HRGC-MS: J&W DB-5 (30 m x 0.25 mm i.d.; film thickness 0.25 μm), SIM-detection (*m/z* 124).

Evidence for the action of two types of carotenoid cleavage enzymes in starfruit

Fig. 10 summarizes the hypothetic breakdown of starfruit carotenoids. Based on previous studies (*34*), a highly specific dioxygenase is expected to cleave epoxycarotenoids (9-cis-violaxanthin or 9'-cis-neoxanthin) across the 11,12 (11',12') double bond to form ABA-derivatives **26-28**. As by-product of ABA biosynthesis formation of isomeric marmelo lactones **23a/b** takes place, the latter being derived from the central portion of the carotenoid chain, which is left after the cleavage of the C_{15}-endgroup (pathway B). By the action of a separate enzyme (pathway A), a generation of C_{13}-norisoprenoids can be rationalized. Although regioselective for the 9,10 (9',10') bond, this enzyme seems to accept various carotenoids, e.g. lutein, violaxanthin, neoxanthin etc., thus resulting in a formation of the primary degradation products shown in Fig. 10, i.e. 3-hydroxy-ß-ionone **3**, 3-hydroxy-5,6-epoxy-ß-ionone **15**, and grasshopper ketone **13**. In addition, oxidative cleavage of ABA-derivatives may result in a formation of 3-oxo-norisoprenoids as has been shown *in vitro* for the conversion of ABA to dehydrovomifoliol **29**.



(A) C_{13}- cleavage products:



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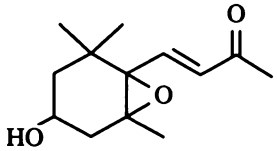

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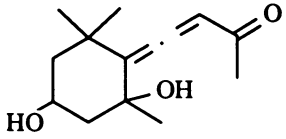
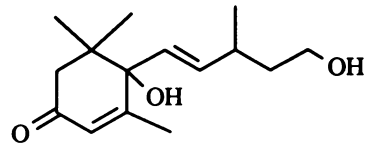


13

C_{13}-cleavage enzyme:

- **regioselective (C_9-C_{10} double bond)**
- **accepts various carotenoids (Lutein, Violaxanthin, Neoxanthin etc.)**

(B) ABA- cleavage products:



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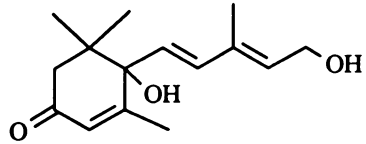

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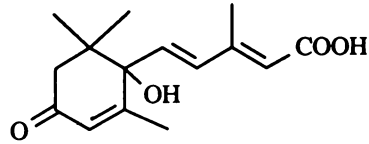

28

ABA-cleavage enzyme:

- **regioselectively (C_{11}-C_{12} double bond)**
- **substrate specific (only epoxy-carotenoids)**

Figure 10. Postulated degradation of starfruit carotenoids rationalizing the formation of C_{13}- and C_{15}-breakdown products.

Due to the importance of carotenoid-derived volatiles for the flavor of many fruits, more attention must be directed in the future to the biodegradation of carotenoids in order to finally elucidate the nature of the responsible cleavage enzymes.

Acknowledgements. Dr. G.K. Skouroumounis, University of Adelaide, South Australia, is thanked for a generous sample of triol **14**. The Deutsche Forschungsgemeinschaft, Bonn, is thanked for financial support.

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Chapter 11

In Vivo and In Vitro Flavor Studies of *Vitis labruscana* Cv. Concord

Kenneth B. Shure and Terry E. Acree

Department of Food Science and Technology, New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456

The development of 4 important odor active compounds in Concord grapes was monitored throughout the growing season in developing berries and in the leaves. These were methyl anthranilate, o-aminoacetophenone, methyl furaneol, β-damascenone and its glycosidic precursor. Callus cultures developed from the immature petiole and fruit tissue of Concord grapes were also analyzed for the presence of these compounds. Only the glycosidic precursor to β-damascenone was present in the callus tissue.

The flavor of Concord grapes and other American grapes of the *Vitis labruscana* and *V. rotundifolia* species make these cultivars unsuitable for the production of most table wines. The grapes possess what has been referred to as labrusca character with foxy and candy like odors (1). This odor character is in strong contrast to the mild aroma of many *V. vinifera* varieties used for the production of popular wines. Ironically it is this same characteristic that makes the Concord grape very popular for the production of non-fermented grape products such as juices and jellies (1).

The source of this foxiness or labrusca character has been examined for many years and can be traced to a few select chemical compounds found only in American grapes and their hybrids. Shown below are methyl anthranilate (2), o-aminoacetophenone (3), furaneol and its methoxy derivative methyl furaneol (4) which, in varying concentrations are responsible for the American character of native grape species.

Concord grapes and other American cultivars also contain β-damascenone (also shown above) and its glycosidic precursor (5,6). This nor-isoprenoid is also found in vinifera grapes (7,8) as well as in other fruits such as apple (9) and tomato (10). β-damascenone and other terpenoid compounds appear to be present in the developing berries while the berries are still green in either free or

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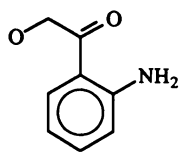
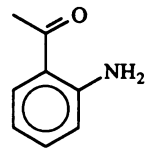
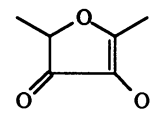
glycosidically bound form (4,12,13). Terpenoid compounds have also been found in the leaves during the growing season (14). On the other hand little information is available regarding the development of the compounds responsible for American character in grape berries. There have been a few studies that have shown that methyl anthranilate increases greatly from the point of veraison (onset of color change in grapes) (15,16) however, to our knowledge, no reports have investigated content prior to veraison or the presence of these compounds in the leaf tissue. In the work described here, three of the American character compounds as well as β-damascenone and its glycosidic precursor were quantitated over the growing season in both berries and leaves to determine if there were differences in where and how these compounds develop in the plant.



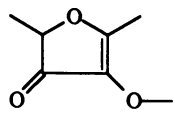
methyl anthranilate



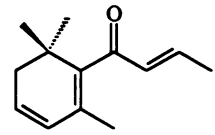
o-aminoacetophenone



furaneol



methyl furaneol



ß-damascenone

In addition to quantitating the American character compounds and β-damascenone and its precursors in berry and leaf tissue throughout the growing season, Concord flavor was investigated utilizing a cell culture system developed from the petioles and immature fruit from Concord vines. The use of undifferentiated plant cell systems for the study of secondary metabolite formation has been described previously (17,18) and in grapes has concentrated on monoterpene (19,20,21) and anthocyanin (22,23,24) metabolism mostly in *vinifera* species.

Experimental

Plant Material - Immature Concord berries and leaves were collected every two weeks beginning mid-July 1992 and continuing until October 7 and extracted for aroma compound analysis. Callus cultures from immature berries and petioles are maintained on Gamborgs B5 media supplemented with 3% sucrose, Gamborgs B5 vitamins, 0.5mg/l 2,4-D, 0.5mg/l BA and 0.8% agar. Callus initiation procedures are described elsewhere (25).

Extraction of Aroma Compounds - Free aroma compounds were extracted using Freon 113 and concentrated by rotary evaporator. Glycosidically bound β-damascenone was trapped using C18 reverse phase adsorbant and eluted with methanol. After drying in vacuo, the precursor was acid hydrolyzed for 20 minutes at pH 2, 90 °C to release β-damascenone which was then extracted with Freon 113. All compounds were quantified using GCMS.

Results and Discussion

The development of flavor in Concord grapes showed large differences between the American character compounds and β-damascenone and its precursor. Some physiological parameters measured over the season can be seen in figure 1. Most noted among these is the absorbance of the juice at 525 nm indicating the development of color in the grape berries. The onset of veraison is clearly shown by the sudden rise around day 58. This is also the point of the sudden rise in concentration of the American character compounds which were undetectable prior to this point (Figure 2). In contrast to this, β-damascenone and its glycosidic precursor were present before as well as after veraison and experienced a less accelerated rise in concentration after the ripening process began (Figure 3). β-damascenone and its glycoside, unlike the American character compounds, were also present in the leaf tissue (Figure 4).

A distinction between the American character compounds and β-damascenone and its glycosidic precursor was also seen in the undifferentiated callus initiated from the Concord grapevine tissue. None of the American character compounds, nor free β-damascenone were present in either the fruit or the petiole callus. The acid hydrolyzable precursor to β-damascenone however, was present in both callus types. The levels were in fact quite comparable to what was found in the intact fruit nearing maturity (26). Later experiments revealed that the level of precursor could be raised either by incubating under a light source or by increasing the level of sucrose in the media (27). A doubling of the sucrose concentration to 6% resulted in levels of bound β-damascenone that surpassed the total β-damascenone content seen previously for Concord grapes (1). Increases in anthocyanin content have been seen in undifferentiated tissue culture of strawberry (28) and grapes (24) when subjected to similar conditions.

These results indicate differences between the development of β-damascenone precursor and the American character compounds. Specifically the β-damascenone precursors are present in immature berries and vegetative tissue whereas the American character compounds were not. Similarly, the undifferentiated tissue produced β-damascenone precursor and not the American character compounds. However, anthocyanins, which are usually absent from immature fruit, can be produced in callus and cell suspensions of grapes. Therefore, the presence of a compound in immature fruit or vegetative tissue is not a prerequisite for production in undifferentiated cells.

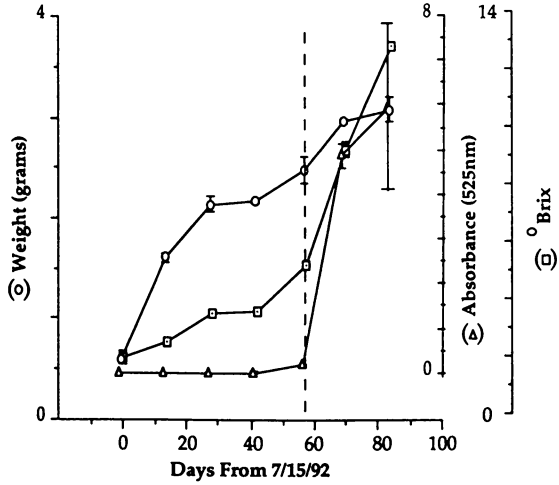


Figure 1 - Physical measurements of berry development over the 1992 growing season. Vertical dashed line in all graphs represents the point of approximate veraison onset.

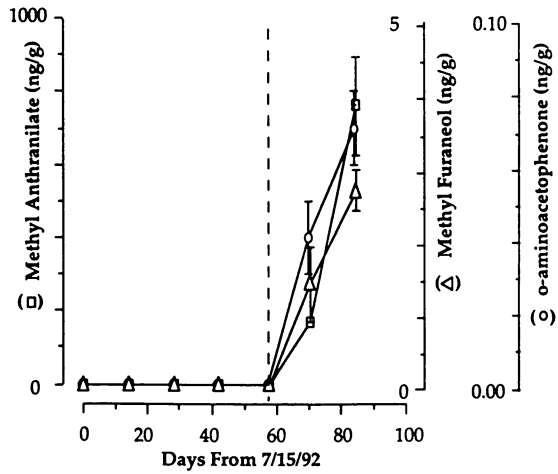


Figure 2 - Measurements of American character impact compounds over the 1992 growing season.

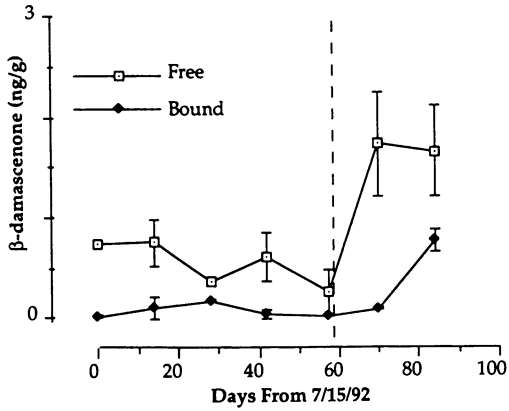


Figure 3 - Measurements of free and bound β-damascenone in the berries over the 1992 growing season.

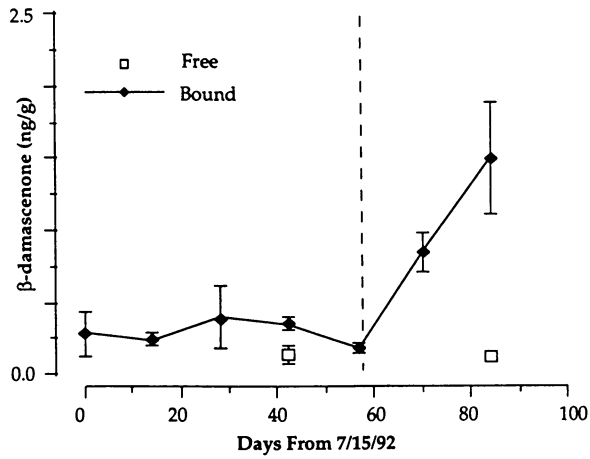


Figure 4 - Measurements of free and bound β-damascenone in the leaves over the 1992 growing season.

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Chapter 12

Substrate Specificity of Alcohol Acyltransferase from Strawberry and Banana Fruits

Jose M. Olias, Carlos Sanz, J. J. Rios, and Ana G. Pérez

Unidad Estructural de Investigación, Fisiologia y Tecnologia Post-recolección, Instituto de la Grasa y sus Derivados, Consejo Superior de Investigaciones Cientificas, Apartado 1078, 41012-Sevilla, Spain

Esters are qualitatively and quantitatively one of the most important class of volatile compounds in fruit aroma. Alcohol acyltransferase plays a major role in the biosynthesis of volatile esters. Proteins with acyltransferase activity from strawberry and banana were partially purified. The enzymes were extracted from separated cells prepared by pectinase treatment, and purified by ammonium sulphate fractionation and gel filtration. The enzymes were tested for their preferences in using different acyl-CoAs and alcohols. Maximum activity for strawberry enzyme was obtained using acetyl-CoA and hexyl alcohol as substrates, and acetyl-CoA and butyl alcohol were the preferred substrates for the banana enzyme. A clear correlation was observed between substrate preference and volatile esters present in both fruits.

Alcohol acyltransferase (AAT) plays a major role in the biosynthesis of volatile esters, not only in fruits but also in the process of fermentation by microorganisms. This enzyme catalyzes the transfer of an acyl moiety from acyl-CoA onto the corresponding alcohol.

Ester formation by microorganisms has been the subject of many reports (1-4). Two alcohol acetyltransferases have been identified and characterized in *Cladosporium cladosporioides* and brewer's yeast (5,6), and an AAT has been purified from Neurospora sp. (7).

Although esters are qualitatively and quantitatively one of the most important class of volatile compounds in fruit aroma, there are very few reports on the biochemical aspects of ester formation in fruits. Yamashita et al. (8) reported the formation of esters from aldehydes that were incubated with whole strawberry fruits. Ueda and Ogata (9) studied the esterification of added alcohols in separated cells of banana, strawberry and melon. The same authors found out that formation of volatile esters in banana fruit was a coenzyme A dependent

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reaction (10). In a recent study (11), we described the purification method and some properties of strawberry AAT. Protein with alcohol acyltransferase activity was purified about 29-fold from Chandler strawberry fruits by ammonium sulphate fractionation, gel filtration and ion exchange chromatography. The enzyme with an apparent Mr of 70 KDa, had a pH optimum of 8.0, and an optimum temperature of 35°C. The unique enzyme previously studied in relation to the esterification process in fruits was the acetyltransferase from banana (12).

The aroma of strawberry and banana has been extensively studied; both fruits have shown different composition in volatile esters. The main volatile compounds in banana aroma are short esters: acetates, propanoates and butanoates (13,14); while in strawberry aroma, the main components are those formed from volatile organic acids with an even carbon number such as acetic, butanoic and hexanoic acid (15,16,17).

Figure 1 summarizes the possible pathways involved in ester formation in fruits. As can be concluded from this scheme, two main factors could determine volatile esters composition in fruits: the availability of the substrates acyl-CoAs and alcohols and the inherited properties of the AAT enzyme (e.g. substrate specificity).

Amino acids metabolism, in relation to aroma biogenesis, has been previously studied in banana (18,19). Radioactive labeling studies have proved that the amino acids valine, leucine and isoleucine, are transformed into branched-chain alcohols, 2-propanol, isoamyl alcohol and 2-methylbutanol, respectively. Tressl and Drawert (19) reported that leucine concentration increases about 3-fold following the climacteric rise in banana. This high level of free leucine available would yield high levels of isoamyl alcohol and, subsequently, high levels of isoamyl esters. Similarly, in a previous work, we studied the possible role of amino acids as precursors in the biogenesis of alcohols in Chandler strawberry (20). In this study, only minor amounts of leucine and isoleucine were found in this fruit and alanine was identified as the main free amino acid. The amino acid profile found in each fruit could explain a different distribution of precursors, alcohols and acids, for volatile ester biosynthesis. Nevertheless, the specificity of enzymes implicated in this biosynthetic pathway leading to ester formation could also have physiological relevance. In this paper, we study the substrate specificity of AAT as a possible factor that could determine volatile ester composition in fruits.

Experimental

Materials. Mature fresh fruits of *Fragaria ananassa* x Duchessne var. Chandler were obtained from Cooperativa Agricola de Lucena del Puerto, Huelva, Spain. Banana fruits, var. Cavendish, were purchased from a local whole market, Mercasevilla, Seville, Spain.

Preparation of crude extract. 250 g of fruits were cut into slices (2 mm thick) and infiltrated with 500 mL of a 0.7 M glycerol solution containing 0.7% pectinase, 0.5% potassium Dextran sulfate and 50 mM mercaptoethanol (ME). After 1 hr agitation at 259°C and filtration through 2 layers of gauze, the separated cells were collected by centrifugation at 3500 x g for 7 min and washed twice with Tris-HCl

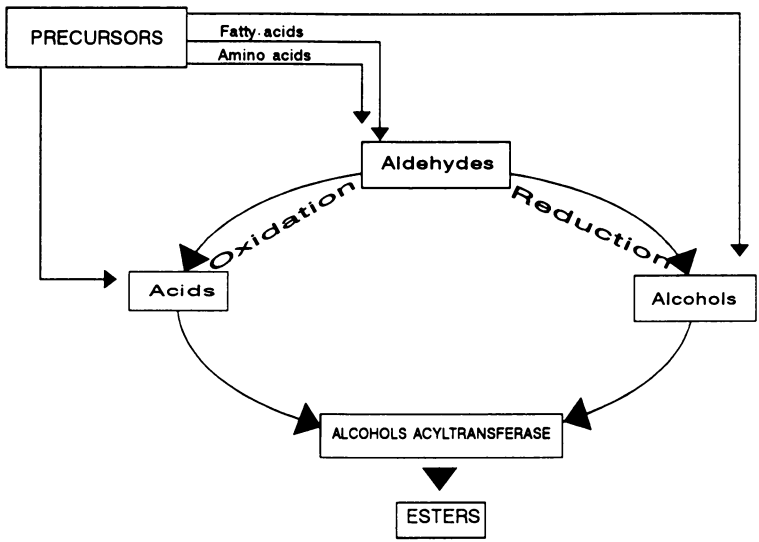

Figure 1. Possible biochemical pathways implicated in volatile ester formation in fruits.

50 mM, pH 8.0, 50 mM ME. Cells were suspended in the later buffer containing 0.1% Triton X100 and disrupted by sonic oscillation (Sonics Mod. 375 W) at 0-4QC for 3 min. The resulting homogenate was centrifuged at 12000 x g for 30 min. The supernatant was considered as the crude extract.

Purification. All procedures were performed at 4°C. Proteins with AAT activity were purified as follows.

Step 1. $(NH_4)_2SO_4$ was added to the crude extract from strawberry and banana tissue and the fraction obtained at 20-70% saturation was collected and desalted on a Sephadex G-25 (Pharmacia) column, using 20 mM Tris-HCl pH 8.0 buffer containing 1 mM dithiothreitol (DTT), 12 mM ME and 10% ethylene glycol (conditioning buffer).

Step 2. The desalted $(NH_4)_2SO_4$ pellet was applied to a Sephacryl S-200 (65 cm x 1.6 cm, Pharmacia) column and eluted with the conditioning buffer. Active fractions were pooled and the resulting enzymatic solution was used for the substrate specificity assays.

Assay of AAT activity. The standard assay mixture consisted of 5 mM Tris-HCl buffer, pH 8.0, 0.25 mM acetyl-CoA, 0.05 mM DTT, 20 mM butyl alcohol, 5 mg BSA and the appropriated volume of enzyme solution (10-50 μL) in a total volume of 0.5 mL (12). The mixture was incubated at 35°C for 30 min in a 11 mL sealed vial. The vial was then transferred into an automatic headspace sampler (Hewlett Packard 19395 A) where a 15 min equilibrium time at 80°C was set to allow the produced ester to enter the gas phase. The reaction product, butyl acetate, was determined by GLC in a gas chromatograph equipped with FID and a stainless steel FFAP (2 m x 2 mm) column at 120°C. The amount of ester was calculated from a calibration curve in the range 3 to 750 nmol. One unit of AAT activity was defined as the amount of enzyme forming 1 μmol of butyl acetate per min.

Results and discussion

The enzymes strawberry AAT and banana AAT were tested for their preference in using different acyl-CoAs and alcohols. In order to examine the reactivities with acetyl-CoA, equal concentrations of various alcohols (20 mM) were added to the standard assay mixture instead of butyl alcohol. Results obtained for both enzymes showed, for all the alcohols tested, higher esterification rate by the enzyme from strawberry. The high levels of esterase activity, enzyme which catalyzes the reverse reaction of AAT in all fruits tissues is the main difficulty for the study of AAT in fruits. Both activities the esterifying activity and the esterase activity have been previously described in isolated cells from strawberry and banana (9). In the same study, the highest values of esterase activity were found in banana cells. This would explain the lower alcohol acyltransferase activity detected in banana extracts.

As it is shown in Table I, strawberry AAT showed maximum activity with hexyl alcohol as substrate. Moreover, the enzyme seemed to be more active against strain-chain alcohols (e.g. amyl alcohol) than against branched chain alcohols (e.g. isoamyl alcohol) of the same carbon number. In the same way unsaturated C_6-alcohol showed light esterification rate.

Table I. Substrate specificity of AAT from strawberry. Comparison of esterification activity with different alcohols, using acetyl-CoA as acyl donor and two different incubation times

	Incubation time	
Ester	30 min	60 min
Butyl acetate	24.3*	37.6
Amyl acetate	22.1	28.0
Isoamyl acetate	17.5	22.5
Hexyl acetate	42.4	52.8
3-hexenyl acetate	3.9	7.3

* nmol of ester formed

All alcohols tested are present as acetate esters in Chandler aroma (Table II). Among all the acetate esters obtained by enzymatic reaction, hexyl acetate (4.5%) is the most abundant in strawberry aroma, followed by butyl acetate (1.9%) and by amyl and isoamyl acetate (1%). A clear correlation between ester composition and alcohol specificity was found.

Table II. Acetate esters in the dynamic headspace of strawberry and banana fruits. Data from references 17,20

Acetate esters	Banana (Var. Cavendish)	Strawberry (Var. Chandler)
Ethyl acetate	9.26*	8.7
Isobutyl acetate	160.4	--
Butyl acetate	38.7	26.2
2-Pentenyl acetate	176.2	--
Isoamyl acetate	257.4	15.3
3-Hexenyl acetate	2.5	4.6
Hexyl acetate	--	61.5
Total volatiles	1384.2	1379.4

* Amounts expressed as ng/g Fruit/80 L of dynamic headspace.

The enzyme from banana showed different esterification rates (Table III). Butyl alcohol was the preferred substrate for banana AAT, followed by amyl and isoamyl alcohol. While the strawberry enzyme showed a clear preference for hexyl alcohol as substrate, assays with banana AAT yielded almost equal amounts of butyl, amyl and isoamyl acetates. As reported for strawberry enzyme, banana AAT showed higher esterification rates against strain chain and saturated alcohols. In the same way, the lowest esterification rate was found for 3-hexenyl alcohol.

Table III. Substrate specificity of AAT from banana fruit. Comparison of esterification activity with different alcohols, using acetyl CoAs as acyl donor and two different incubation times

	Incubation time	
Ester	30 min	60 min
Butyl acetate	11.2*	24.7
Amyl acetate	12.9	23.5
Isoamyl acetate	10.5	18.7
Hexyl acetate	5.7	16.0
3-hexenyl acetate	3.1	5.3

* nmol of ester formed.

These results can also be explained according to the volatile composition of banana, in which isoamyl acetate is considered to be a character impact compound. As is shown in Table II, isoamyl acetate is the main acetate ester found in this fruit. In the same way, the low activity exhibited for banana AAT with hexyl alcohols as substrates could explain the low amount of these esters identified in this fruit.

A similar experiment was carried out in order to determine the acyl-CoA preference of both enzymes: equal concentrations (0.25 mM) of different acyl-CoAs were assayed using butyl alcohol as the co-substrate. Results obtained using acetyl, propyl and butyl-CoAs are shown in Table IV.

Table IV. Substrate specificity of AAT from strawberry and banana fruit. Comparison of esterification activity with different acyl CoAs, using butyl alcohol as the co-substrate, 60 min as incubation time

Ester	Strawberry	Banana
Butyl acetate	34.0*	19.1
Butyl propionate	7.0	2.5
Butyl butanoate	27.0	3.6

* nmol of ester formed.

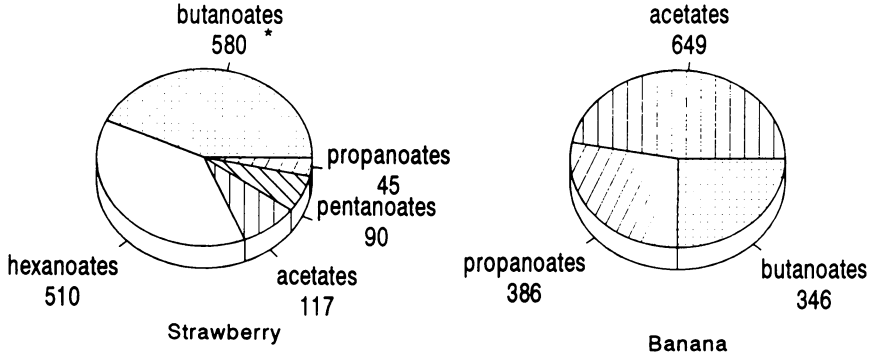

Figure 2. Volatile esters distribution in the headspace of strawberry and banana. Data from references 17,20. (*) Amounts expressed as ng/g fruit/80 L of dynamic headspace.

Both enzymes showed the highest activity using acetyl-CoA as substrate, although butyl propanoate and butyl butanoate were also formed. The relative activities exhibited by strawberry and banana AAT with propyl and butyl-CoA were clearly different. Strawberry AAT showed a high activity with butyl-CoA (80%) compared to the poor esterification rate obtained for the same acyl-CoA using the enzyme from banana (20%). Banana AAT seems to be a more selective enzyme forming only very low amounts of butyl propionate and butyl butanoate.

Although we have not examined these two enzymes against higher acyl-CoAs, results obtained could have physiological relevance, since they are in good agreement with the acyl moieties distribution in the volatile ester composition of strawberry and banana (Figure 2).

Thus, AAT from strawberry showed less specificity for propyl-CoA and propionate esters were detected as minor components (3%) in the dynamic headspace of Chandler. In the same way, higher selectivity for butyl-CoA corresponds with higher amount of butanoates (43%) in Chandler aroma. Same correlation is observed in banana in which acetates are the main group of esters, representing 13 half of the total identified compounds in banana aroma, and AAT showed the maximum activity with acetyl-CoA.

Conclusions

Although further work should be done, data obtained for strawberry and banana AAT allow us to suggest that substrate specificity of AAT enzyme could be a key factor in aroma biogenesis of fruits.

The knowledge about enzymes catalyzing the biosynthesis of fruit aroma compounds, such as volatile esters, is not only interesting from a biochemical point of view but is an essential basis for future improvement of natural aroma and flavor by genetical engineering of plants.

Acknowledgments

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Chapter 13

Bioconversion of Citrus *d*-Limonene

R. J. Braddock[1] and K. R. Cadwallader[2]

[1]Institute of Food and Agricultural Sciences, Citrus Research and Education Center, University of Florida, 700 Experimental Station Road, Lake Alfred, FL 33850
[2]Department of Food Science and Technology, Mississippi State University, Box 9805, 112 Herzer Building, Mississippi State, MS 39762

Microbiological or enzymatic conversion of terpenes to value-added compounds is increasingly important because of consumers' preference for "natural" flavors. Microbes and enzymes capable of using terpenes such as limonene as growth media and substrates have been identified and studied. One such enzyme, α-terpineol dehydratase (αTD), purified > 10-fold after cell-disruption of *Pseudomonas gladioli*, stereospecifically converted (4R)-(+)-limonene to (4R)-(+)-α-terpineol or (4S)-(-)-limonene to (4S)-(-)-α-terpineol. αTD also showed stereoselectivity, since the hydration rate of (4R)-(+)-limonene was approximately 10X the rate of hydration of (4S)-(-)-limonene. Presence of αTD is believed to be common among psuedomonads. For example, a strain of *P. cepacia*, closely related to *P. gladioli*, was found capable of converting limonene to α-terpineol. Other pseudomonads have also been isolated that perform this conversion.

The terpene, (4R)-(+)-4-isopropenyl-1-methylcyclohexene (CA Registry No. 5989275), (+)-limonene, commonly called d-limonene, is the major constituent of citrus essential oils (approximately 95% (v/v) in oils from orange and grapefruit peel). Some chemical and physical properties and material safety data for (+)-limonene have been compiled and published (*1*).

The primary sources of (+)-limonene are in raw materials generated during processing of citrus fruit for juice. It is decanted from evaporator condensate streams during concentration of peel press liquor to molasses. It is steam distilled from emulsions during cold-pressed peel oil processing and is also recovered as a product of essential oil concentration (*2*). (+)-Limonene is utilized as a raw material for chemical syntheses of terpene resin adhesives and value-added flavor chemicals. Other applications include solvent uses in waterless hand cleaners, pet shampoo and degreasing agents (*3*). Annual world availability of (+)-limonene amounts to about 50 million kg and is primarily dependent on

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the amount of oranges processed in the major citrus growing regions of Florida and Brazil (*4*).

The perfume and flavor industries find many applications for monoterpenes derived from (+)-limonene and similar terpenes. Many are oxygenated compounds which can be chemically synthesized using various process conditions. However, such chemical processes may result in non-stereospecific, uneconomic or undesirable end-products. Since terpene flavor compounds are synthesized by a variety of microorganisms and higher plants, there is considerable interest in using biological processes to produce metabolites of value for flavoring foods.

Bioconversion of terpenes includes use of plant cells in suspension to convert valencene to nootkatone (*5*) as well as tissue culture techniques. Knorr et al. (*6*) described the bioconversion of (+)-limonene to carvone by dill cultures. Such biotechnological methods applied to flavor production have been the subject of many reports and were reviewed by Schreier (*7*).

Microbial Conversions

Bacteria and fungi may differ in their metabolism of terpenes. If one considers (+)-limonene, bacteria generally metabolize by progressive oxidation starting with the 7-methyl group. Generation of small amounts of neutral products which are not further metabolized may occur. Fungi attack (+)-limonene by hydration of the double bond of the isopropenyl substituent or by epoxidation-hydrolysis of the 1,2 double bond. Some of the terpene products identified during various microbial conversions of (+)-limonene are illustrated in Figure 1. Many of these compounds, in addition to (+)-limonene, have economic value as flavoring ingredients and odorants.

Other Terpenes

The subject of microbial terpene conversions has been reported in the literature by a number of authors who studied compounds other than (+)-limonene. The degradation of (+)-camphor using a pseudomonad isolated from sewage resulted in 2,5-diketocamphane and other products (*8*). Prema and Bhattacharyya (*9*) transformed α- and ß-pinene with *Aspergillis niger* and isolated optically pure (+)-verbenone and (+)-cis-verbenol. ß-Pinene was also transformed by *Pseudomonas pseudomonallai* to camphor, borneol, α-terpineol and similar compounds (*10*). Transformation by *Botrytis cinerea* of citronellol, a significant terpene of grape must, resulted in production of several diols, depending on growth conditions (*11*). A patent was issued for bacterial decomposition of geraniol and citronellol from citrus processing waste streams using *Pseudomonas* strains (*12*).

Limonene

(+)-Limonene has been the subject for many microbial bioconversion studies, because of its abundance and chemistry. Research to improve the quality of citrus oils resulted in the discovery that bacterial growth in peel oil emulsions reduced the quantity of (+)-limonene, increasing the concentration of α-terpineol

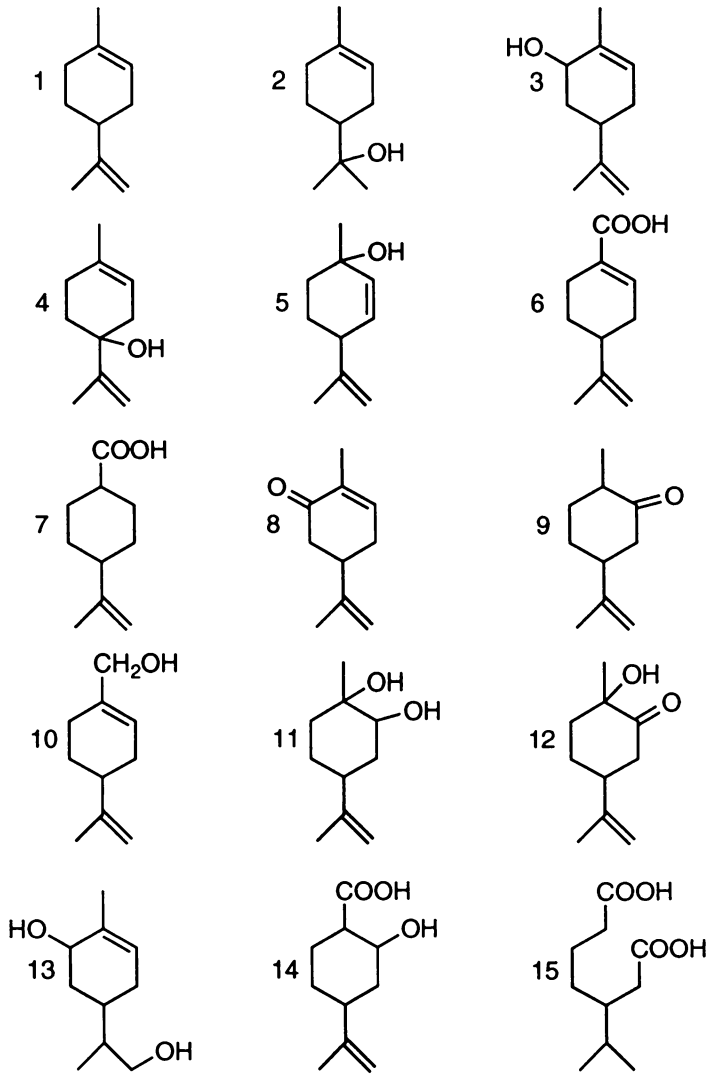

Figure 1. Chemical structures (1-15) of some products resulting from the microbial transformation of limonene. (1) limonene, (2) α-terpineol, (3) carveol, (4) p-mentha-1,8-dien-4-ol, (5) p-mentha-2,8-dien-1-ol, (6) perillic acid, (7) dihydroperillic acid, (8) carvone, (9) dihydrocarvone, (10) perillyl alcohol, (11)p-menth-8-ene-1,2-diol,(12)p-menth-8-ene-1-ol-2-one,(13)p-menth-1-ene-6,9-diol, (14) 2-hydroxy-p-menth-8-ene-7-oic acid, (15) ß-isopropenyl pimelic acid.

(*13*). A bacterium capable of conversion of (+)-limonene to perillic acid was isolated (*14*). *Cladosporium* (*15*) as well as *Diplodia* and *Corynespora* (*16*) were capable of converting (+)-limonene into 1,2-diols. Bowen (*17*) isolated *Penicillium* strains that could transform (+)-limonene to several products, including p-menthadienol, carveol, carvone and perillyl alcohol. The pathway has been defined for *Pseudomonas incognita* conversion of (+)-limonene to perillic acid and ß-isopropenyl pimelic acid (*18*).

The bacterium, *Pseudomonas gladioli*, isolated from pine bark and sap was shown to metabolize (+)-limonene as a sole carbon source, producing (+)-α-terpineol and (+)-perillic acid (*19*). In this study, concentrations of (+)-limonene as high as 10% (v/v) had no apparent toxic effect on the organism's growth; however, a considerable lag period was observed. This lag phase was speculated to be a result of the organism conditioning to the growth media. Optimum growth conditions (highest growth rate and maximum cell concentration) occurred near 1% (v/v) concentration of (+)-limonene. The organism produced the limonene bioconversion products, (+)-perillic acid and (+)-α-terpineol, illustrated in Figure 2. The (+)-perillic acid, after increasing, was metabolized during extended growth of the organism; however, (+)-α-terpineol increased and maintained a constant concentration.

In addition to *P. gladioli*, other pseudomonads have been isolated that covert limonene to α-terpineol. A strain of *P. cepacia*, capable of metabolizing either α- or ß-pinene as sole carbon source, was isolated from soil (*5*). The bacterium exhibited optimum growth at 30°C and pH 5.5, and grew equally well in media containing from 1 to 5% (v/v) α- or ß-pinene. Major bioconversion products from α-pinene included borneol, p-cymene, α-terpinolene, limonene, camphor, terpinen-4-ol, α-terpineol and p-cymene-8-ol. Bioconversion products from ß-pinene were similar except for the presence of fenchyl alcohol and absence of camphor.

Precursor studies have indicated that limonene was an intermediate product, readily metabolized as sole carbon source by the bacterium (*6*) to form perillic acid as major product and a trace amount of α-terpineol. In addition to *P. cepacia*, several other soil pseudomonads give a similar product profile from limonene (*7*).

A recent study has shown that (+)-limonene can be converted by the mold *Aspergillus cellulosae* in sugar media containing limonene to (+)-limonene diol, (+)-carveol, (+)-perillyl alcohol and (+)-isopiperitenone (*20*). A review of many microbial conversions of (+)-limonene leading to products for the flavor and perfume industries has also been published (*21*).

Enzymatic Conversions

Enzymes have a long history of being used as processing aids in the food and flavor industries. Major enzymes and uses have been reviewed (*22*). Enzymes capable of terpene bioconversions have been isolated from some microorganisms. An aldehyde dehydrogenase catalyzing conversion of perillyl aldehyde to the acid was isolated from a soil pseudomonad which utilized limonene as a carbon source (*23*). This enzyme was active towards a number of aldehydes including phellandral, benzaldehyde and acetaldehyde. A typical flavor conversion

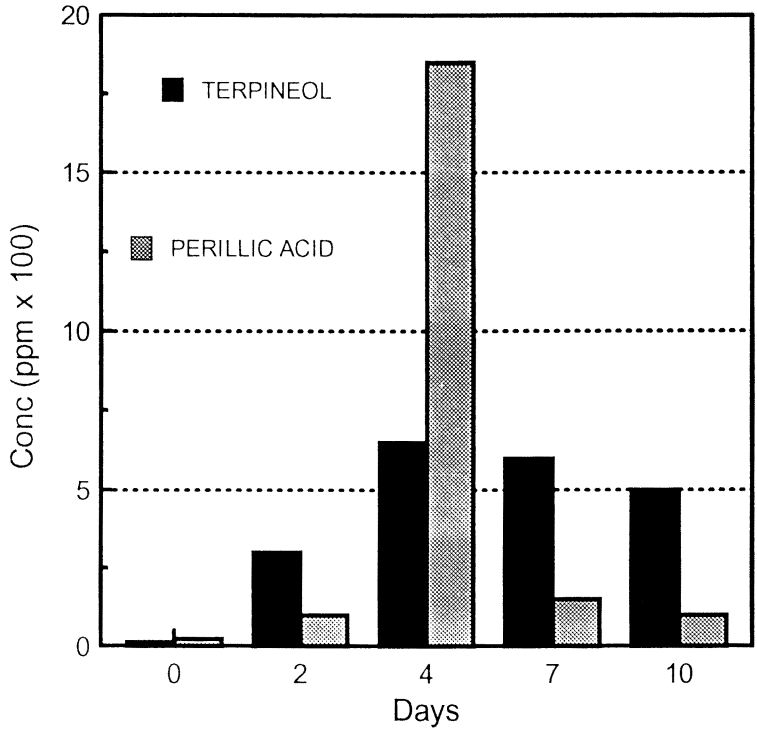

Figure 2. Concentrations of α-terpineol and perillic acid vs. growth time in (+)-limonene and mineral salts of the organism *Pseudomonas gladioli*.

mediated by alcohol dehydrogenase is the production of geranial from geraniol (*24*).

An enzyme, α-terpineol dehydratase (αTD), has been isolated from *P. gladioli*, partially solubilized and characterized (*25*). This enzyme might also be described as Δ-8,9-limonene hydratase. αTD stereospecifically converted (4R)-(+)-limonene to (4R)-(+)-α-terpineol or (4S)-(-)-limonene to (4S)-(-)-α-terpineol. αTD also showed stereoselectivity, since the hydration rate of (4R)-(+)-limonene was approximately 10X the rate of hydration of (4S)-(-)-limonene. Kinetic studies of the formation of (+) and (-)-α-terpineol from racemic limonene were followed using chiral gas chromatography (*26*). The relative amounts of each enantiomer at various reaction times are presented in Table I. The relative percent concentration of (4R)-(+)-α-terpineol increased at a faster rate than (4S)-(-)-α-terpineol, indicating stereoselectivity for the (+)-limonene substrate.

Table I. Relative Percent Concentration of α-Terpineol Enantiomers as a Function of Time for the Hydration of 0.2 mM Racemic Limonene by α-Terpineol Dehydratase at 25°C

	Relative Percent Concentration[a]	
Time (hr)	*(4S)-(-)-α-terpineol*	*(4R)-(+)-α-terpineol*
0	0.0	0.0
5	10.8	89.2
10	11.3	88.7
20	11.4	88.6
40	13.3	86.7

[a]Relative percent conc. = $\frac{\text{Conc. of pure } \alpha\text{-terpineol enantiomers}}{\text{Total conc. of } \alpha\text{-terpineol}}$

The aforementioned studies demonstrate the considerable potential of bioconversion processes for the transformation of inexpensive monoterpene precursors, such as limonene, into more valuable flavor and fragrance compounds. The majority of these investigations were concerned with the elucidation of pathways for limonene metabolism; however, a few of these researchers recognized the potential of using bioconversions for the generation of novel and more valuable flavor and fragrance compounds. The real advantage of a biotechnological process over a synthetic process may not necessarily be in its greater selectivity and specificity, or its ability to produce rare or novel compounds, rather the fact that it is a natural process and thus generates natural products. This is especially important currently, when consumers prefer "natural" flavors and fragrances over their "synthetic" or "artificial" counterparts.

Acknowledgments

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Chapter 14

Ester Biosynthesis in Relation to Harvest Maturity and Controlled-Atmosphere Storage of Apples

John K. Fellman[1] and James P. Mattheis[2]

[1]Department of Plant, Soil, and Entomological Sciences, University of Idaho, Moscow, ID 83843
[2]Tree Fruit Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Wenatchee, WA 98801

Volatile esters from acids and alcohols are important components of flavor and odor perception in apples. The relationship between alcohol acetyl CoA transferase (AAT) acetate ester-forming activity, non-ethylene volatile emission, and flesh volatile content of apples during the maturation period and after removal from controlled-atmosphere (CA) storage was investigated. At the appropriate times, apples were sampled for volatile compounds in the headspace and flesh using solid sorbent along with purge-and-trap capillary gas chromatography. Development of a spectrophotometric assay for AAT activity using 5,5'-dithiobis-(2-nitrobenzoic acid) to react with free CoA liberated in the esterification reaction is reported. Subsequently, acetate ester forming activity was assayed on partially-purified extracts of cortical tissue. Ester forming activity is apparently sensitive to oxygen atmospheres as well as stage of harvest maturity. Upon removal from low-oxygen atmospheres, AAT activity is initially depressed but recovers to levels approaching that of air-stored controls. Flesh concentrations of acetate esters, major contributors to apple flavor, are related to levels of enzymatic activity in the tissue. It is important to identify preclimacteric harvest windows that include information concerning the biosynthesis of odor and flavor volatile components in order to ensure maximum quality attributes in apples stored for long periods of time.

There is little information concerning biochemical studies of flavor molecule synthesis in fruits, particularly apples. Traditionally, research with apples has concentrated on easily measurable quality attributes such as firmness, acidity, and color. There are many reports of the relationship

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between harvest maturity and long term storability of apples in controlled atmospheres (CA). For apples grown in the Pacific Northwest region of the USA, commercial producers use established procedures to assess proper harvest maturity to ensure year-round availability of quality fruits (1,2). There has been increasing interest in investigating aspects of harvest maturity and storage with relation to nature, occurrence and concentration of volatile compounds responsible for perception of aroma and flavor (3). In related research, our previous studies with 'Bisbee Delicious' apples demonstrated the relationship between emission of certain volatile compounds and fruit harvest maturity (Figure 1).

Our previous experiments with 'Delicious' apples placed in anaerobic atmospheres showed a marked increased in emission of ethyl acetate at the expense of other esters seen when fruit is stored in air (Table I).

Table I. Some Esters Emanating from 'Delicious' Apples when Stored for 30 Days in an Anaerobic Atmosphere Compared to Normal Controlled-Atmosphere Conditions

Ester	Before storage	1.5% O_2 storage	0.05% O_2 storage
ethyl acetate[a]	0	0	26.7
butyl acetate	0.3	0.2	0.2
2-methyl-1-butyl acetate	2.5	1.7	0.7
ethyl butyrate	0	0	1.5
ethyl propanoate	0	0	0.9
ethyl 2-methyl butyrate	0	0	2.8
ethyl hexanoate	0	0	0.6
ethyl octanoate	0	0	0.1

[a]values are from fruit on day 1 post storage, expressed as $nL^{-1}kg^{-1}h^{-1}$. Adapted from reference 23.

The family of acetate esters are major contributors to the perception of fruit flavor. Butyl acetate, 2-methyl-1-butyl acetate, and ethyl acetate have reported olfactory thresholds 0.066, 0.005, and 5.0 ppm v/v (4), respectively. Most studies to date report the effects of CA storage on volatile production and emission (5,6,7,8,9,10), yet there is a lack of information regarding the nature of the enzymes responsible for synthesis of some flavor molecules. The enzyme responsible for acetate ester biosynthesis, alcohol acetyl CoA transferase (EC 2.3.1.84, abbreviated

AAT), has been reported in fruits (11,12,13). The relationship between content of acetate esters and acetate ester-forming activity of apples during the maturation period and after removal from CA storage was investigated.

Materials and Methods

Plant material and storage conditions. 'Rome' apples were harvested at weekly intervals in 1992 from a block (planted in 1976) located in a commercial orchard near Fruitland ID, USA. After transportation to the laboratory, the bulk of each apple sample was immediately placed in refrigerated (0-1°C)(RA) or 0-1°C controlled-atmosphere (CA) storage at 0.5% v/v oxygen/1% CO_2 with subsamples placed in chambers to measure carbon dioxide and ethylene evolution. Measurement of the time lag between harvest and initiation of the respiratory climacteric ("Green life") was accomplished by placement of fruit samples in small chambers and electronically monitoring CO_2 and ethylene production (14). Fresh or stored fruits were sampled for volatile compounds in the headspace and flesh, as well as AAT activity. Fresh apples were assayed one and six days after harvest. Apples were removed from storage and placed in the laboratory under prevailing conditions of heat and light (approximately 25°C; 12 h photoperiod), and assayed after one and six days post-storage.

Volatile compound analysis. Headspace volatiles were measured by placing fruits in polytetrafluroethylene jars, passing purified air through the jar at 100 ml/min for 10 min to collect 1 L of headspace atmosphere on 50 mg of Tenax, a porous polyester substrate. Trapped volatiles were thermally desorbed into a cryofocusing loop and analyzed as described below. All associated connective tubing was made of polytetrafluroethylene to ensure inertness. To assay volatile content of flesh, mesocarp tissue was crushed and 2.5 ml of juice diluted 1:1 with distilled deionized water was analyzed using purge-and-trap cryofocusing techniques. Samples were purged in a closed system for 5 min with helium, and water vapor condensed from the sampling stream by passing the vapors through a cryostat held at -10°C. Samples were injected by cryofocusing at -90°C using a commercial purge-and-trap injector (Chrompack International B.V. Middelburg, The Netherlands) modeled after that reported by Badings *et al.* (15). Gas chromatographic separations were achieved using conditions reported by Mattheis *et al.* (16), but the DB-WAX column diameter was 0.32 mm with 5.0 μ film thickness. Quantitation was achieved using flame-ionization detection. Positive identification of volatile molecules was facilitated by interfacing the gas chromatograph to a Hewlett-Packard 5971 Quadrupole Mass Spectrometer operated in the electron ionization mode at 70eV. Identification was via Wiley/NIST library match and injection of standard compounds.

Extraction of proteins. Seventy-five g of cortical tissue from three 'Rome' apples was ground with polyvinyl pyrollidone powder (1.25 g/10 g tissue) in 0.1 M phosphate buffer pH 7.0 and centrifuged at 16,000 x g for 30 min. Ammonium sulfate was added to 80% saturation of the decanted supernatant. After stirring for 1 h at 0-4°C, the extract was centrifuged, and the precipitate dialyzed against pH 7.0 phosphate buffer at 0-4°C overnight. The dialysate was separated from the precipitate (residual carbohydrate polymers) and used for enzyme assays. This procedure typically resulted in a 8-10 fold purification of enzymatic activity measured on a protein basis. Protein was measured using a commercial dye-binding kit (Biorad Laboratories CA USA) following the method of Bradford (17).

Assay of Alcohol Acetyl CoA Transferase (AAT). Esterification of butanol to butyl acetate was measured by incubating partially-purified protein extracts of apple tissue in the presence of acetyl coenzyme A and butanol. Assay components in final concentrations were: 10 mM potassium phosphate buffer pH 7.0, 20 mM $MgCl_2$, 1 mM 5,5' dithiobis-nitrobenzoic acid (DTNB, Ellman's reagent) in pH 7.0 phosphate buffer, 3 mM 1-butanol, and 0.49 mM Acetyl CoA. Reactions were assayed by mixing ingredients in a 1 ml cuvette, and initiating the reaction with acetyl CoA. Progress of the esterification reaction was followed by monitoring solution absorbance at 412 nm, the absorbance maximum of the thiophenol product formed by reaction of DTNB with free CoA. This is essentially the procedure reported by Fellman *et al.* (18).

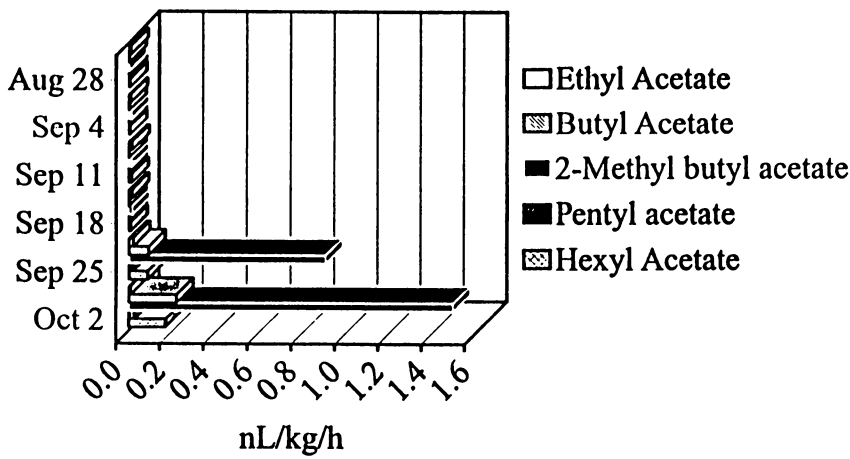

Figure 1. Changes in acetate esters emanating from 'Bisbee Delicious' apples during physiological development for the 1990 harvest season. Figure adapted from data in reference 15.

Results and discussion

The respiration patterns of 'Rome' apples harvested in 1992 indicated the relative ages of apples used in these experiments (data not shown). Harvest 1 was considered to be at the onset of the climacteric; harvest 2 mid-climacteric, and harvest 3 late or post-climacteric. Climatic conditions in 1992 caused an earlier than normal bloom period resulting in unusually early apple maturation in Idaho.

Esters formed in relation to harvest maturity. Major types of esters found in headspace and flesh of 'Rome' apples are listed in Table II.

Table II. Nature and Occurrence of Esters Measured in Flesh and Headspace of 'Rome' Apples

Compound	Flesh	Headspace
Ethyl Acetate	x	x
Propyl acetate	x	
Butyl Acetate	x	x
2-Methyl-butyl acetate	x	x
Hexyl Acetate	x	x
Ethyl Propanoate		x
Ethyl 2-methyl butyrate		x
Hexyl Propanoate		x
Hexyl Butyrate		x

It is interesting to note the absence of certain esters in the flesh and the presence of certain esters in headspace alone. This implies synthesis of some compounds is peel related, while others are made in the cortical tissue as well. In this study, we concentrated on occurrence of acetate esters in flesh tissue as measured by purge-and-trap GC. Figures 2, 3, and 4 show total acetate esters in relation to harvest maturity and length of time in CA or Refrigerated air storage.

In fresh apples, ester concentration increased with advancing harvest date except in the '262' variety, where total ester content slightly decreased between harvest 1 and 3. Fresh apple ester content increased when apples were allowed to stand at room temperature for 6 days (Figure 2). The major ester components of fresh 'Law Rome' apples were butyl acetate and 2-methyl butyl acetate, comprising 65-85 % of the

total flesh-purgeable acetate esters. Fresh '262 Rome' apples had similar ester characteristics, but there was more 2-methyl butyl acetate than any other compound (data not shown).

Esters formed during refrigerated and CA storage. Such was not the case when apples were stored for 3 months (Figure 3). Ester content dropped during the 6 day post-storage period regardless of storage atmosphere. At day 1, 3 months in 0.5% oxygen, ethyl acetate comprised the major acetate ester measured. This phenomenon was previously observed in 'Delicious' apples held in anaerobic atmospheres for 30 days (12). Ester concentrations in 'Rome' apples were comparatively lower after 6 months storage, again regardless of the storage conditions. The drop in ester content during the post-storage period was less at 6 months compared to 3 months (Figure 4). Apparently, ester content is a ripening related phenomenon, as evidenced by the continuous increase during the harvest

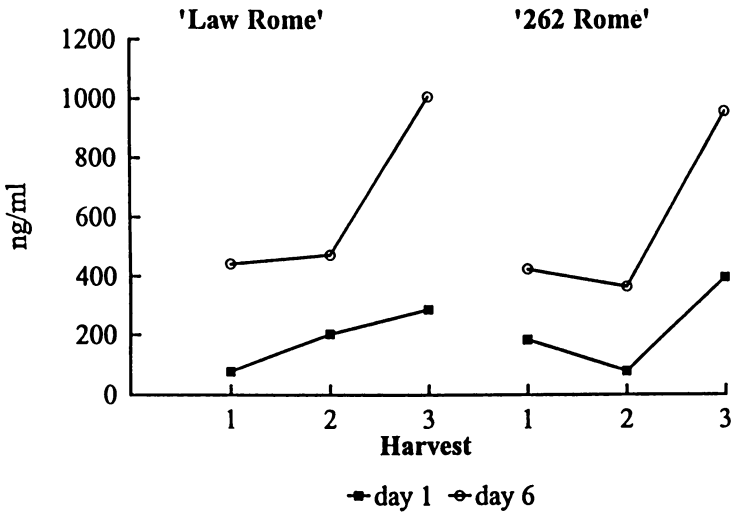

Figure 2. Total acetate ester content of fresh 'Rome' apple flesh in relation to harvest maturity (x-axis). Harvest 1, 2, and 3 are considered early, mid-, and postclimacteric stages, respectively.

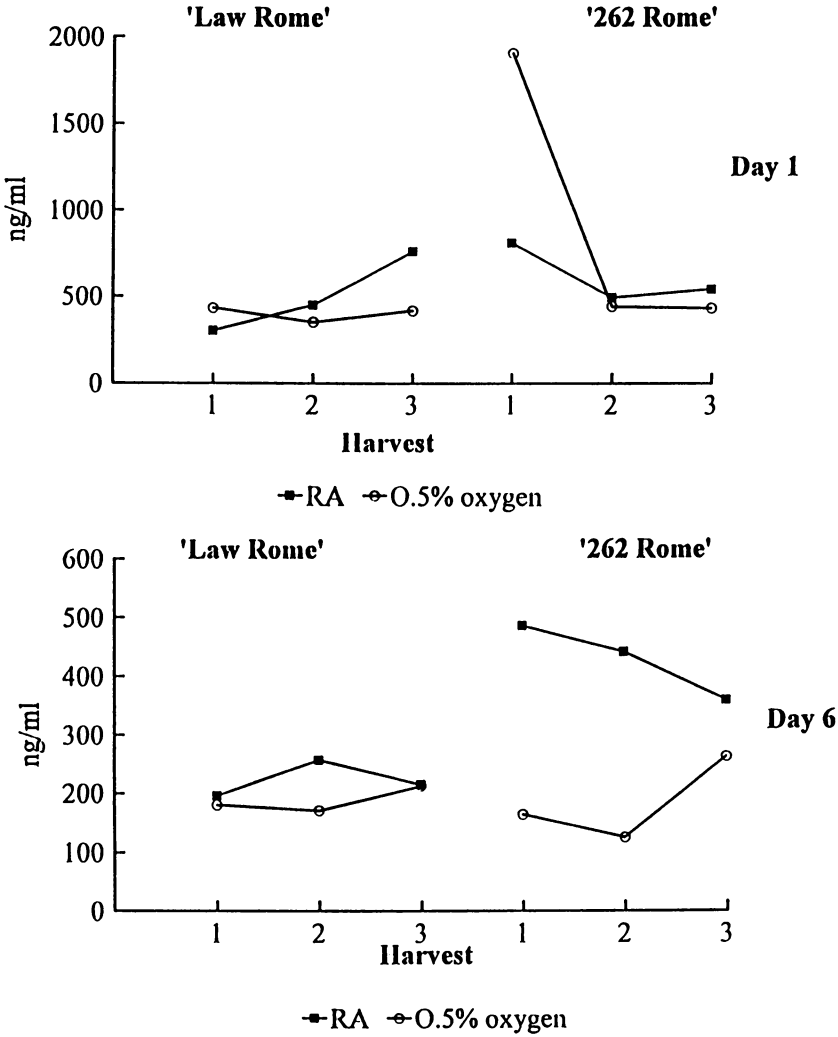

Figure 3. Total acetate ester content of 'Rome' apple flesh in relation to harvest maturity (x-axis), storage atmosphere (squares = RA; open circles = CA), and ripening period (1 or 6 days) out of storage after 3 months.

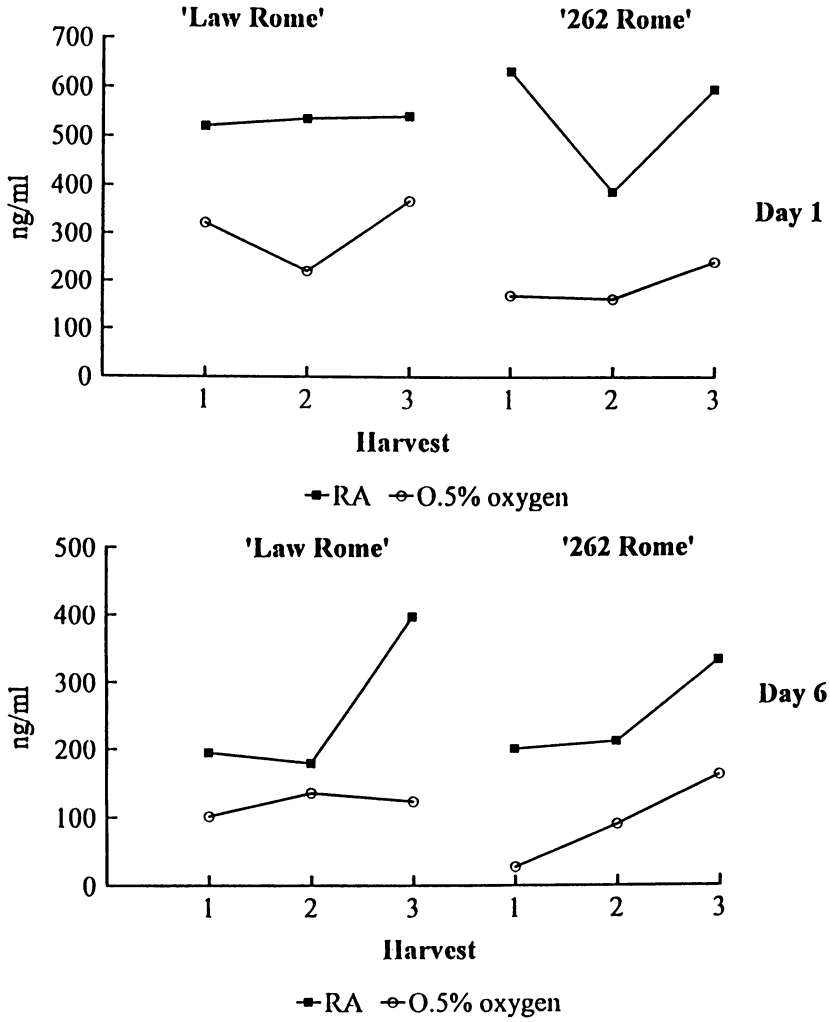

Figure 4. Total acetate ester content of 'Rome' apple flesh in relation to harvest maturity (x-axis), storage atmosphere (squares = RA; open circles = CA), and ripening period (1 or 6 days) out of storage after 6 months.

period, as the climacteric ripening phase advanced. 'Delicious' apples exhibit the same type of pattern (16).

Ester content, while high when apples are initially removed from storage, decreased rapidly during the post storage ripening period, suggesting some factor limiting regeneration. Since ester synthesis has been observed when apples are treated with alcohol vapors (19,20) it is quite likely that availability of the alcohol substrate limits the nature and amount of volatile flavor compounds found in apple flesh subjected to various storage conditions. Branched-chain amino acids are the precursors for volatile alcohols with valine identified as the major precursor for methyl butanol and heptanol (21). Amino acids, the putative precursors for some esters are known to decrease in concentration during ripening and remain relatively constant in storage (22).

Acetate ester biosynthesis. Formation of butyl acetate as measured in the reaction mixture by purge-and-trap capillary GC was proportional to the amount of thiophenol generated in the reaction mixture.(Figure 5). Stoichiometry for our assay is not 1:1. i.e., there is approximately 2 moles thiophenol formed per mole butyl acetate produced over the two minute time course of assay. The activities we report for AAT are comparatively higher than that reported by other investigations in other fruits (11,14). Possible explanations include, a requirement by the AAT enzyme to bind an additional Acetyl CoA molecule for catalytic activity, or the method of assay(measurement of acetyl CoA hydrolysis) eliminates the error introduced by measurement of the end product, butyl acetate. It is likely that measurement of the end-product ester is not accurate, due to the copious presence of esterase activity in fruits (13,23). Enzyme activity measurement is best made as initial velocity, not at equilibrium conditions. This study compared levels of activity in relation to different stages of fruit maturity and storage regimes.

Capacity for enzymatic ester synthesis paralleled that of fresh fruit in relation to harvest timing. Figures 6 and 7 show the results of AAT assays performed on purified extracts of apples in relation to harvest maturity and various lengths of storage in refrigerated air or 0.5% oxygen atmospheres. The day 1 values were coincident with the ester content measured in the apple flesh. However in the 262 variety of Rome, day 6 enzyme activity values decreased for the last harvest, again suggesting the association between ester biosynthesis and ripening, i.e. ester formation follows climacteric status closely. In Law Rome, a higher coloring variety, AAT activity continued to rise as harvest and ripening advanced. When apples were held in refrigerated storage, capacity for ester biosynthesis changed dramatically. In the 262 variety, 3 month values paralleled that of fresh fruit; the activity patterns resembled those seen at harvest. At 6 months refrigerated storage, ester biosynthesis capacity substantially decreased, although the relationship between day 1, day 6, and harvest time values remain relatively consistent. Law Rome

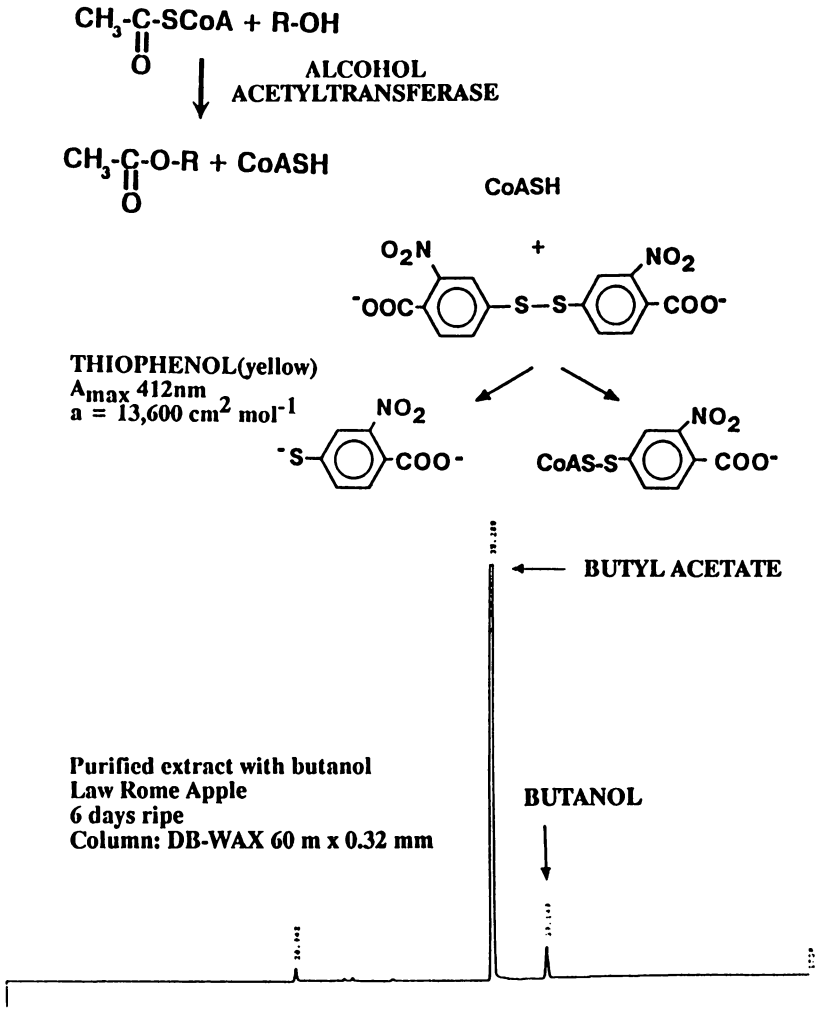

Figure 5. Development of a spectrophotometric assay for alcohol acetyltransferase. TOP: Enzyme catalyzed reaction; MIDDLE: Reaction of free Coenzyme A with reagent; BOTTOM: Purge-and-trap GC analysis of reaction performed on 'Rome' apple extract.

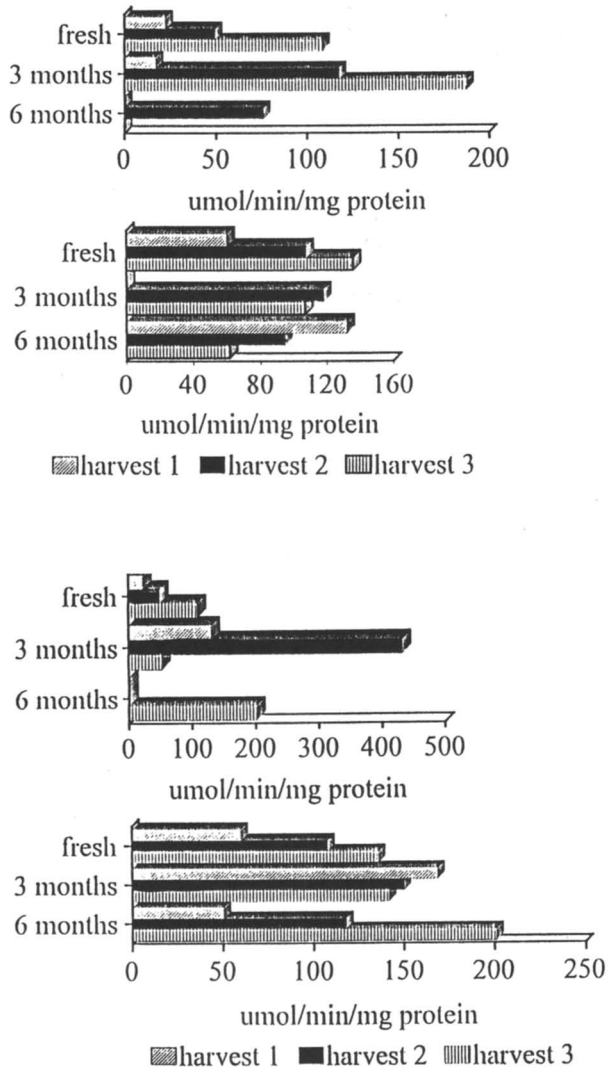

Figure 6. AAT enzyme activity of 'Law Rome' apple extracts. TOP: Refrigerated air storage removed and assayed at day 1 and day 6 after storage; BOTTOM: Controlled-atmosphere (0.5% oxygen) storage removed and assayed at day 1 and day 6 after storage.

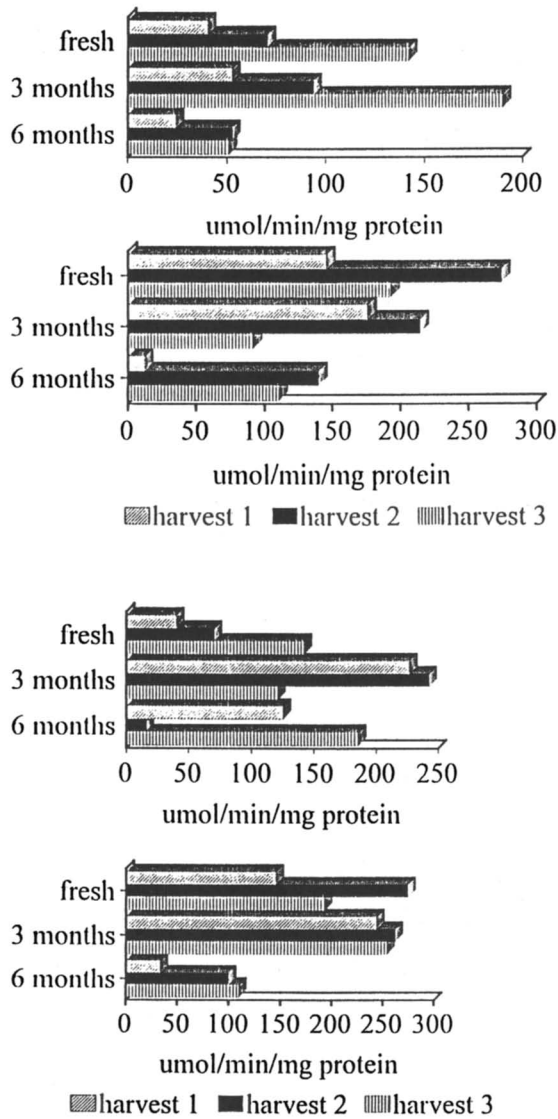

Figure 7. AAT enzyme activity of '262 Rome' apple extracts. TOP: Removed from refrigerated air storage and assayed at day 1 and day 6 after storage; BOTTOM: Removed from controlled-atmosphere (0.5% oxygen) storage and assayed at day 1 and day 6 after storage.

AAT values at 3 months refrigerated storage were similar to those of fresh apples, but by 6 months had decreased. It is interesting to note the trend of increased activity with advancing harvest date had reversed after 6 months RA storage.

Apples held in 0.5% oxygen refrigerated storage showed patterns of AAT activity different than those observed in RA storage. Values for 262 Rome were similar to fresh fruit after 3 months in CA. Between 3 and 6 months CA storage, capacity for ester biosynthesis decreased markedly. CA-stored Law Rome AAT values resembled those of fresh fruit, especially those observed after 6 months storage. In contrast to RA fruit, where after 6 months there was a trend of decreasing activity, there was an increase in AAT activity in relation to advancing harvest maturity, more like the trend observed in fresh fruit.

Acetate esters are important descriptors of apple flavor and odor. The enzyme responsible for synthesis of acetate esters, alcohol acetyl CoA transferase EC 2.3.1.84, has been partially purified and remains active in apple flesh under various storage conditions. At harvest, ester content and enzyme activity continued to rise concomitant with the increase in climacteric respiration. This suggests that in order for flavor to develop adequately, compromises must be made for harvest timing in relation to length of storage. Most studies with apples report a loss in other quality attributes such as firmness and acidity when harvest is delayed past the onset of the respiratory climacteric. This study demonstrated postclimacteric 'Rome' apples to have the most acetate ester content at harvest and after 6 months storage. Flavor molecule synthesis appeared to increase with ripeness, and in every case was higher in air-stored fruit after a 6 day ripening period. Low oxygen CA storage seemed to decrease ester content and enzymatic activity responsible for ester biosynthesis, especially during periods longer than 3 months. It may be possible to manipulate the oxygen level in CA storages in order to preserve the ester synthesis capacity of the tissue, once the genes encoding for ester synthetic enzymes have been expressed during fruit ontogeny. After a period out of storage dependent upon initial conditions, the enzymatic activity returns to a level approaching that of air-stored fruit. The maximum amount of activity occurs earlier in fruit removed from low-oxygen storage after having lower initial levels compared to RA fruit. It is unknown whether this protein is synthesized *de novo* or is reactivated by exposure to ambient oxygen, but the enzyme data suggest reactivation since we did not observe enzymatic activity to be substantially greater than that seen in RA fruit. Future studies are directed at investigating this phenomenon.

Acknowledgements

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Chapter 15

Studies on Tomato Glycosides

Gerhard E. Krammer[1], Ron G. Buttery, and Gary R. Takeoka

U.S. Department of Agriculture, Agricultural Research Service, Western Regional Research Center, 800 Buchanan Street, Albany, CA 94710

The volatile flavor compounds of tomatoes (*Lycopersicon esculentum*, Mill.) have been studied extensively. Recent research work on flavor precursors in tomatoes has shown the presence of various flavor compounds in bound form. There is only very limited data available on the various forms of tomato flavor conjugates. Furthermore, there is some evidence that the glycosidic fraction obtained from tomato fruits consists of compounds with nonvolatile aglycons. In the past, different techniques of countercurrent chromatography (CCC) have been used successfully for the separation of flavor precursors from complex natural matrices. The application of different CCC-techniques to the purification of glycosidic extracts from the tomato plant and elucidation of the structure of isolated compounds by means of NMR-spectroscopy and mass spectrometry are discussed.

Previous studies on the volatile compounds in fresh tomatoes (*1*) and tomato paste (*2*) helped to elucidate the characteristic aroma of fresh and processed tomatoes. Using acid and enzyme mediated hydrolysis, the bound volatiles of fresh tomatoes have been studied (*3-5*). Different studies on flavor precursors in grapes (*6*), sour cherries (*7*), apples (*8, 9*), strawberries (*10, 11*), raspberries (*12-14*) and quince fruit (*15*) have also shown the presence of glycosidic progenitors. In tomatoes, Fleuriet and Macheix (*16*) identified the glucosides of p-coumaric acid, caffeic acid, ferulic acid and sinapic acid. The aim of the present work was the isolation and structure

[1]Current address: Haarmann & Reimer GmbH, Corporate Research, Postfach 1253, D-37601 Holzminden, Germany

elucidation of glycoconjugates of flavor compounds in tomatoes. In recent years modern countercurrent chromatographic techniques (CCC) were successfully used for the isolation of polar plant consituents such as glycosides (*17*). Therefore, we decided to apply three different CCC instruments for the separation of glycosidic extracts from tomatoes.

EXPERIMENTAL SECTION

Plant Material: Freeze concentrated tomato serum (*L. esculentum*) was obtained from a major U.S. tomato processor using processing type tomatoes related to cultivars such as FM785 and GS-12.

Chemicals: HPLC grade methanol, 1-butanol, 1-propanol and diethyl ether were obtained from Fisher Scientific Co. (Springfield, NJ, USA). Diethyl ether was distilled before use. 2-Pentanol was obtained from Lancaster Synthesis Inc. (Windham, NH, USA) and from Aldrich Chemical Co. (Milwaukee, WI, USA). Amberlite XAD-2 (20-60 mesh) non ionic polymeric adsorbent was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The resin was cleaned by continuous extraction in a Soxhlet apparatus with methanol refluxing for 8 h.

Isolation of a Glycosidic Extract. After 2 kg of freeze concentrated tomato serum was filtered, the aqueous solution was divided into two equal portions. Each fraction was applied to an Amberlite XAD-2 column (25 x 700 mm, 10 mL/min) (*18*). After the column was washed with 8000 mL of distilled water, a glycosidic extract was obtained by desorption with 1 L of methanol. The methanol eluate was concentrated under reduced pressure and afterwards freeze-dried (yield 5 g).

Enzymes. Almond β-glucosidase and hesperidinase (from *Aspergillus niger*) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The pectinase Cytolase PCL5 was provided by Genencor Inc. (South San Francisco, CA, USA).

Enzyme Mediated Hydrolysis. The freeze-dried and preseparated glycosidic extracts (between 4 mg and 10 mg) were dissolved in 5 mL 0.2 M phosphate buffer (pH=5.0). After adding almond β-glucosidase (2 mg), hesperidinase (20 mg) or Cytolase PCL5 (50 μL), respectively, the mixture was incubated at 37 °C overnight. The liberated aglycons were extracted with diethyl ether, dried (anhydrous Na_2SO_4) and concentrated. The extract was subjected to HRGC and HRGC-MS analysis. For every sample a blank test without addition of enzyme was carried out.

NMR-Spectroscopy. NMR spectra were taken on a Bruker 400 ARX spectrometer. The Bruker Jmod pulse sequence was used.

DCI Mass Spectrometry. Electron impact and direct inlet chemical ionization mass spectra were recorded on a double focusing VG 70/70-HS mass spectrometer, Fisons Inc. (Manchester, U.K.).

Capillary Gas Chromatography-Mass Spectrometry (GC-MS). A HP 5890 Series II gas chromatograph equipped with a split/splitless injector was connected by direct coupling to a HP 5971 Mass Selective Detector. A J&W DB-1 (60m x 0.25mm i.d., df = 0.25 μm) was used with the following temperature program: 35 °C (4 min isothermal), raised to 225 °C at 4 °C/min and held at 225 °C for 15 min. The

injection mode was splitless (1 min). The temperature of the injector was 190 °C and the temperature of the transfer line 220 °C. The mass spectrometer was operated in scan mode over a mass range from 35 to 350 m/z.

High Performance Liquid Chromatography (HPLC). For HPLC analysis a HP Series 1050 quaternary pump fitted with a Rheodyne (Cotati, CA, USA) sample injector (20 μL) and a Varex evaporative light scattering detector (ELSDII), Varex (Rockville, MD, USA) was used. The light scattering detector was operated at a drift tube temperature of 150 °C and at an exhaust temperature of 80 °C (range =1, time const. = 0.1). The gas pressure was 25 psi nitrogen. The HPLC analysis of the preseparated fraction was performed on an Econosil C-18, 4.6 x 250 mm, 10 μm, column, Alltech Assoc. (Deerfield, IL, USA). The eluant was methanol-water (20 : 80 % to 80 : 20 % in 25 min) with a flow of 1 mL/min.

Droplet Countercurrent Chromatograph (DCCC). A Droplet Countercurrent Chromatograph model DCC-A from Tokyo Rikakikai Co., Ltd. equipped with 288 glass tubes (400 mm x 2 mm i.d.) was used. The instrument was operated at 25 °C in the ascending mode with the upper layer from a $CHCl_3$/methanol/H_2O (7:13:8) solvent system as the mobile phase at pressure of approx. 320 psi. The flow rate was 8 mL/h and a 10 mL glass injection chamber was used. During every run 42 fractions (4 mL each) were collected.

High Performance Centrifugal Partition Chromatograph (HPCPC). A Series 1000 High Performance Centrifugal Partition Chromatograph from Sanki Laboratories, Inc. (Mount Laurel, NJ, USA) equipped with 2 partition disk packs (2136 partition channels) was operated at room temperature. The partition disks are engraved with 1.5 x 0.28 x 0.21 cm channels connected in series by 1.5 x 0.1 x 0.1 cm ducts (*19*). The lower layer of 1-butanol/1-propanol/H_2O (4:1:5) was used as the mobile phase (descending mode). The adjusted rotor speed was 1000 rpm. The upper layer of this solvent system was used as a stationary phase. The flow rate of 8 mL/min was provided by a Gilson Model 305 pump (Model 25 SC pump head) equipped with a 806 manometric module, Gilson Medical Electronics Inc. (Middleton, WI, USA) and a Rheodyne model 7161 sample injector (2 mL sample loop). The system was operated at a pressure of 550 psi. For online UV monitoring a Gilson Model 115 UV detector was used. The fractions were collected with a ISCO Foxy 200, ISCO (Lincoln, NE, USA) fraction collector at 1 min intervals.

High Speed Countercurrent Chromatograph (HSCCC). A High Speed Countercurrent Chromatograph Model CCC-1000 from PHARMA-TECH Research Corp. (Baltimore, MD, USA) equipped with three coil columns (Tefzel tubing 1.6 mm i.d., total volume 325 mL) was operated at room temperature. For the purification of the preseparated samples a 2-pentanol/water (1:1) solvent system was applied using the lower phase as a mobile phase. The upper phase of this solvent system served as the stationary phase. The solvent changing valve was set to the "head to tail" mode. The sample was introduced by a Rheodyne Type 50 teflon rotary valve with 2 mL sample loop. The flow rate was 1 mL/min (Gilson Model 305 pump, cf. HPCPC). The system was operated at a pressure of 80 psi. For the online monitoring of the effluent a HP series 1050 variable wavelength detector with

preparative flowcell was used. The sampling rate of the fraction collector (cf. HPCPC) was 5 min/vial.

RESULTS AND DISCUSSION

Countercurrent Chromatography. Countercurrent Chromatography (CCC) is defined as a form of liquid-liquid chromatography. In CCC systems either centrifugal or gravitational force is employed to maintain a bed of one liquid phase, while a stream of a second immiscible phase is passed through the system by a pump having contact with the stationary phase (*20*). Since no solid supporting matrix is involved, the separation process is done without any effect of adsorption chromatography. As a result of this, the retention time of a solute in the system depends only on the phase volume ratio and the partition coefficient in the particular solvent system. The partition coefficient (K) is the most important term in CCC. It is defined as

$$K = \frac{C_s}{C_m} = \frac{\text{Conc. of solute in stationary phase}}{\text{Conc. of solute in mobile phase}} \quad (1)$$

Once the partition coefficient is known the retention time (R_t) of a solute in a given solvent system can be predicted using the following equation.

$$R_t = \frac{V_s * K + V_m}{\text{Flow}} \quad (2)$$

K: Partition coefficient
R_t: Retention time (min)
V_s: Volume of the stationary phase (mL)
V_m: Volume of the mobile phase (mL)
Flow: Flow rate of mobile phase (mL/min)

The volume of the stationary phase (V_s) and the volume of the mobile phase (V_m) in the system is determined by a graduated cylinder. For applications with a high performance centrifugal partition chromatograph (HPCPC) the dead volume due to the ducts between every partition channel has to be considered for the determination of the retention time (R_t). The flow rate of the mobile phase is usually determined by the pump. The equation for the standard resolution (R_s) helps to emphasize two major advantages of CCC: Sample Capacity and Selectivity.

$$R_s = \frac{1}{4}(\alpha\text{-}1) * \sqrt{N} * \frac{K}{K + \frac{V_m}{V_s}} \quad (3)$$

Selectivity Efficiency Capacity

Modern countercurrent chromatography apparatus operate at a very high volume of stationary phase (V_S). Therefore, the value of the phase volume ratio V_m/V_S is smaller for CCC systems than for preparative HPLC columns (*21*). As a result the capacity term of equation 3 is large for CCC separations. In this case a large amount of stationary phase directly contributes to an improvement of the resolution (R_S). The number of theoretical plates required to obtain a given resolution is less important in CCC than in HPLC. Therefore, the currently available CCC instruments can operate with good resolution although having a significant smaller number of theoretical plates (350 to 1000) than HPLC columns (*22*). On the other hand, the selectivity term is easily influenced by having a tremendous variety of solvent systems possible. Therefore, for each separation the selection of an optimum solvent system is very important. For the separation of polar and unstable compounds the above principles of countercurrent chromatography are very advantageous.

CCC-systems. The droplet countercurrent chromatograph (DCCC), developed by Tanimura and coworkers (*23*), consists of approximately 300 narrow vertical glass tubes (2 mm i.d.) which are interconnected by Teflon tubing. The stationary phase is maintained in the system by gravity. The mobile phase is passed through the system by a pump, forming a stream of droplets in the stationary phase. The DCCC-apparatus can be operated in the ascending or the descending mode by using either the upper or the lower layer of a biphasic system as a mobile phase, respectively. In order to find a suitable solvent system for DCCC separations we analyzed the glycosidic extract from tomatoes using a thin-layer chromatographic (TLC) method, published by Hostettman (*24*). Finally, the solvent system chloroform/methanol/water (7:13:8) was chosen. This system has already been successfully used for the preseparation of glycosidic extracts from grape juice (*25*) and Riesling wine (*26*). According to the nomenclature of the inventor, Yoichiro Ito (*27*), the DCCC-apparatus and also the high performance centrifugal partition chromatograph (HPCPC), discussed below, are hydrostatic equilibrium systems (HSES).

The HPCPC-apparatus has a stacked circular disk rotor. The partition disks are engraved with 2136 partition channels which are connected in series by ducts. The whole rotor is connected to the injector and detector through two rotary seals. Like the DCCC the HPCPC can also be operated in either ascending or descending modes. The HPCPC is less dependent on the droplet formation of mobile phase in the stationary phase than the DCCC according to the results of Foucault and coworkers (*19*). This research group also showed that the resolution of a HPCPC remains fairly constant over a broad range of flow rates (*28*). This is a major difference compared to a hydrodynamic equilibrium system (HDES) like the High Speed Countercurrent Chromatograph (HSCCC), where higher flow rates can cause a significant decrease of resolution because of the loss of stationary phase.

In HSCCC-systems Teflon or Tefzel tubing is wrapped in many layers around a spool in order to form a so-called "multi-layer coil". In the HSCCC-system we used for our applications three of these multi-layer coils are mounted on a rotating frame that enables a planetary movement of each coil around the center axis of the frame. Since the axis of the coil in this system is parallel to the axis of the whole frame this

type of instrument is referred to as co-axial CCC. In cross-axial CCC, the axis of the coil holder is in a 90 ° position to the axis of the frame. When the multilayer coil rotates around its own axis, the Archimedean screw force tends to carry the stationary phase to one end of the coil which is called the head. During operation the mobile phase is pumped in the opposite direction, which is the "head to tail" direction. The interfacial friction force of the mobile phase and the Archimedean screw force are counteracting and come to dynamic equilibrium, which depends mainly on the rotational speed, flow rate and viscosity of the solvent system (*20*). Therefore, instruments based on this principle are referred to as hydrodynamic equilibrium systems (HDES).

Glycoconjugates in tomatoes. A glycosidic fraction was obtained from 2 kg freeze concentrated tomato serum by using column chromatography on XAD-2 resin. This extract (approx. 5 g) was separated in 3 separation steps on a DCCC-apparatus. The upper layer of a chloroform/methanol/water system (7:13:8) served as a mobile phase. With this solvent system the more polar constituents emerge very early, while the less polar compounds can be found in the later fractions. Each fraction was analyzed off-line by HPLC using a light scattering detector. Fractions with similar chromatographic profile were pooled. Furthermore a small amount of every combined fraction was hydrolyzed by hesperidinase, β-glycosidase and Cytolase PCL5, respectively. The resulting aglycons were extracted and subjected to HRGC-MS analysis. The diagram in figure 1 shows the amount of each combined fraction. The very polar fractions 8-10 and 11-12 represented a very large portion of the whole glycosidic extract which indicated a need for a chromatographic system capable of resolving extremely polar mixtures.

Adenosine. From a less polar fraction (fraction no. 20b) we isolated adenosine (**1**) using the HPCPC instrument with a 1-butanol/1-propanol/water system (4:1:5). This example shows that a screening of aglycons using enzyme or acid hydrolysis does not give a complete picture of the pool of glycoconjugates, since compounds like C- and N-glycosides are not detected with this methodology. For this problem the combination of high performance liquid chromatography with mass spectrometry (HPLC/MS) and especially with tandem mass spectrometry (HPLC/MS/MS) offers multiple solutions. New electrospray ionization techniques give very promising results for the study of glycoconjugates. Recently also negative ion formation in electrospray mass spectrometry of nucleotides and other compound classes has been studied and for aminoglycosides a significant cleavage along the glycosidic bond has been observed (*29*).

p-Coumaric acid β-D-glucopyranoside. The application of the HPCPC-apparatus using the same solvent system (1-butanol/1-propanol/water) for a more polar fraction (fraction no. 15) revealed a partially resolved peak (fig. 2). After two more separation steps under the same chromatographic conditions, p-coumaric acid β-D-glucopyranoside (**2**) was identified using ^{1}H- and ^{13}C-NMR spectroscopy. The molecular weight was determined by chemical ionisation mass spectrometry with

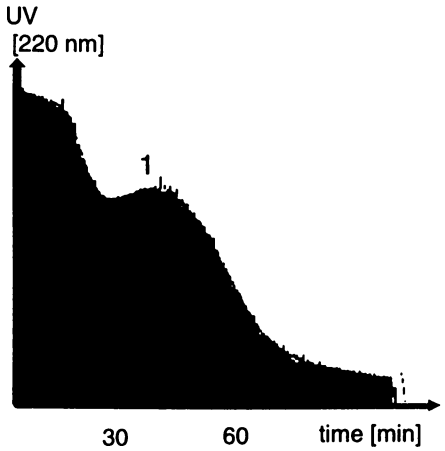


Figure 1. DCCC separation of a glycosidic extract obtained from tomatoes. Result of three separations (total 5 g). Conditions: solvent system $CHCl_3$/methanol/water (7:13:8); mode of operation, ascending (mobile phase = upper phase); flow = 8 mL/h; fractions of 4 mL were collected.

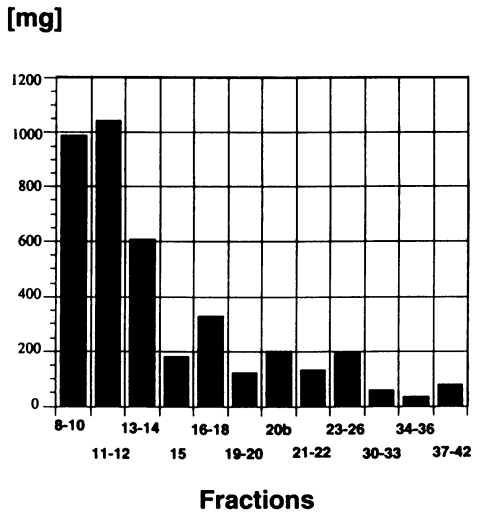
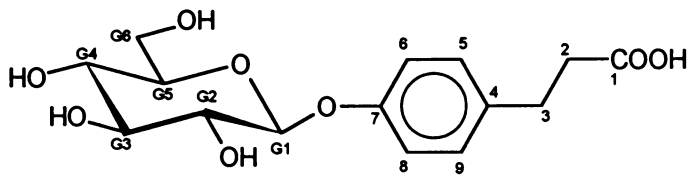


Figure 2. HPCPC separation of p-coumaric acid β-D-glucopyranoside. Conditions: Sample, 185 mg; solvent system, 1-butanol /1-propanol/H_2O (4:1:5); mode, descending (lower phase as mobile phase); stationary phase content, 75%; rotor speed, 1000 rpm; flow, 8 mL/min; pressure, 550 psi.

ammonia yielding a pseudo-molecular ion at 344 m/z ($M+NH_4^+$). After enzyme hydrolysis using hesperidinase, 4-vinylphenol as major decarboxylation product of the 4-hydroxycinnamic acid was found during HRGC/MS analysis. Fleuriet and Macheix (*16*) reported this glucoside as well as the glucose ester of 4-hydroxycinnamic acid to be constituents of tomatoes. They also found that the glucoside was always being present in a larger amount than the corresponding ester.

Operating the HPCPC-instrument at high flow rates (8 mL/min) enabled a relatively fast isolation of p-coumaric acid β-D-glucopyranoside, although no baseline separation was achieved. For the further purification of preseparated DCCC fractions we used the HSCCC-apparatus with a 2-pentanol-water solvent system.

3-(4-Hydroxyphenyl)propionic acid β-D-glucopyranoside. The HSCCC separation of a polar fraction (Fraction no. 16-18) using a 2-pentanol-water solvent system revealed an excellent resolution. The purity of peak 1 (fig. 3) was sufficient for direct structure elucidation using ^1H and ^{13}C NMR spectroscopy (Table I, Table II).



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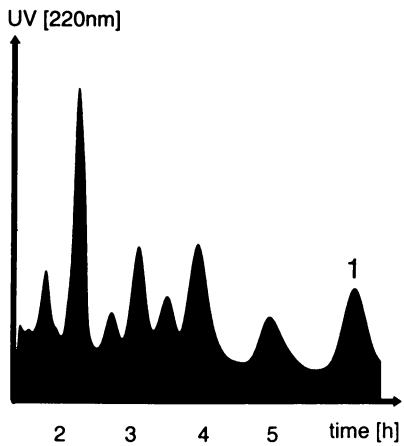


Figure 3. HSCCC separation of 3-(4-hydroxyphenyl)propionic acid β-D-glucopyranoside. Conditions: Sample, 250 mg; solvent system, 2-pentanol/water (1:1); mode, "head to tail" (lower phase = mobile phase); stationary phase content, 70 %; flow, 1 mL/min; pressure: 80 psi.

Table I. ^1H NMR spectral data of the isolated 3-(4-hydroxyphenyl)propionic acid β-D-glucopyranoside (**3**) and of reference compounds (δ relative to TMS, 400 MHz, CD_3OD)

Isolated	glucoside (**3**)		**3a**
Aglycone	moiety		
δ (ppm)	Signal	Assignment	δ (ppm)
2.54	2H, t (7.6)	$H_{a/b}$-2	2.52
2.85	2H, t (7.6)	$H_{a/b}$-3	2.80
7.0	2H, d (8.7)	H-6, H-8	6.70
7.14	2H, d (8.7)	H-5, H-9	7.02
Glucose	moiety		**A**
3.35-3.49	3H, m	H-G2, H-G4, H-G5	3.35-3.69
3.69	1H, br. t (ca. 9)	H-G3	3.35-3.69
3.72	1H, dd (12; 5)	H-$G6_b$	3.68
3.88	1H, dd (12; 2)	H-$G6_a$	3.87
4.85	1H, d (7.5)	H-G-1	n.d.

Coupling constants (J in Hz) in parentheses.
Assignment was done by comparison with reference compounds and published reference data: **3a**, 3-(4-hydroxyphenyl)propionic acid from Aldrich Chemical Comp.; **A**, Glucose moiety of 4-(4'-hydroxyphenyl)-butan-2-one-4'-O-β-D-glucopyranoside (*30*).

The molecular weight of compound **3** was determined by DCI mass spectrometry with ammonia. Prominent ions were obtained at m/z 346 (100), 180 (74), 166 (69), 268 (33), 328 (4). The most abundant ion at m/z 346 corresponding to the characteristic pseudo-molecular ion $[M+NH_4]^+$ whereas the molecular ion at m/z 328 is only detected with lower intensity. At m/z 166 the molecular ion peak of the 3-(4-hydroxyphenyl)propionic acid **(3a)** was observed. The ion at m/z 268 can be explained as the loss of acetic acid from the intact molecule [M - 60]. Using FAB mass spectrometry with a glycerol matrix the ion at m/z 268 was the most abundant one indicating less soft ionisation conditions.

In previous studies, 3-(4-hydroxyphenyl)propionic acid **3a** was tentatively identified after enzyme mediated hydrolysis of a glycosidic extract of tomatoes (*5*). This is the first report of glycoconjugate **3** in tomatoes, which is the dihydro derivative of compound **2** .

Benzyl β-D-glucopyranoside. The HSCCC-separation of a less polar fraction (23-26) showed a fairly complex chromatogram (fig. 4) in the online UV-detection at 220 nm. The GC/MS analysis after enzyme hydrolysis (hesperidinase) of small portions at different sections of the chromatogram led to a fraction containing predominately benzyl alcohol. After freeze drying, the characterization of the isolated compound **4** was done by mass spectrometry as well as ^1H and ^{13}C NMR spectroscopy. In the DCI mass spectrum a pseudo-molecular ion with a relative abundance of 100% was detected at m/z 288 $[M+NH_4]^+$ while the molecular ion at m/z 270 had a relative abundance of 10%. Furthermore, a characteristic ion for hexoses was detected with a relative abundance of 70% at m/z 180 $[Hexose-H_2O+NH_4]^+$. This indicated the presence of a glycoconjugate consisting of a hexose and an aglycone with the molecular weight of 108. In the EI mass spectrum prominent ions were obtained at m/z 91 (100%), 163 (21%), 43 (70%). The high abundance of the benzyl-ion at m/z 91 was congruent with the result of the enzyme hydrolysis yielding benzyl alcohol as an aglycone. The ^1H NMR spectrum (Table III) exhibited a doublet at δ 4.35 (J=7.7 Hz) for one anomeric proton, indicating the presence of a β-glycosidic linkage. Comparing the data obtained from the ^1H- and ^{13}C-NMR spectrum (Table III, Table IV) with published data of authentic reference compounds the isolated glucoside **4** was identified as benzyl β-D-glucopyranoside.

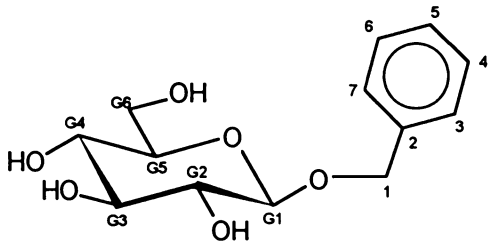


Table II. ^{13}C NMR spectral data (100 MHz, CD_3OD) of the isolated 3-(4-hydroxyphenyl)propionic acid β-D-glucopyranoside (**3**) and of reference compounds.

Isolated	glucoside (**3**)		**3a**
Aglycone	moiety		
Position	δ* (ppm)	Jmod	δ* (ppm)
1	177.48	C	176.99
2	37.60	CH_2	37.13
3	31.78	CH_2	31.18
4	134.48	C	132.91
5[a]	130.74	CH	130.21
6[b]	118.31	CH	116.15
7	158.08	C	156.64
8[b]	118.31	CH	116.15
9[a]	130.74	CH	130.21
Glucose	moiety		**A**
G1	102.96	CH	102.52
G2	75.43	CH	74.94
G3	78.59	CH	78.01
G4	71.88	CH	71.42
G5	78.48	CH	78.12
G6	63.00	CH_2	62.54

* Chemical shifts were assigned on the basis of a Jmod experiment and by comparison with reference substances as well as published data: **3a**, 3-(4-hydroxyphenyl)propionic acid; **A**, Glucose moiety of 4-(4'-hydroxyphenyl)-butan-2-one-4'-O-β-D-glucopyranoside (*30*).
[a-b] interchangable values.

Table III. ^1H NMR spectral data of the isolated benzyl β-D-glucopyranoside (**4**) and of reference compounds (δ relative to TMS, 400 MHz, CD_3OD)

Isolated	glucoside (**4**)		A
Aglycone	moiety		
δ (ppm)	Signal	Assignment	δ (ppm)
4.66	1H, d (11.8)	H_a-1	4.64
4.93	1H, d (11.8)	H_b-1	4.91
7.30	5H, m	H-3, H-4, H-5, H-7	7.31
Glucose	moiety		
3.25-3.5	2H, m	H-G2,H-G4,H-G5	3.42, 3.46, 3.32
3.83	1H, br. t (ca. 9)	H-G3	3.79
3.69	1H, dd (11.8; 5)	H-$G6_b$	3.71
3.89	1H, dd (11.8; 2)	H-$G6_a$	3.88
4.35	1H, d (7.7)	H-G1	4.42

Coupling constants (J in Hz) in parentheses.
Assignments were made with the aid of published reference data: **A**, benzyl β-D-glucopyranoside (*31*).

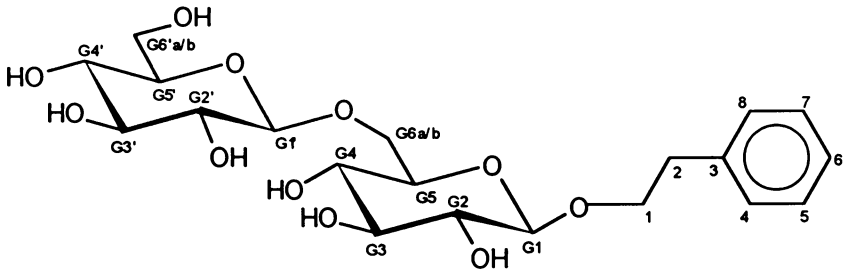
Table IV. ^{13}C NMR spectral data (100 MHz, CD_3OD) of the isolated benzyl β-D-glucopyranoside (**4**) and of reference compounds

Isolated	glucoside (**4**)		A
Aglycone	moiety		
Position	δ* (ppm)	Jmod	δ* (ppm)
1	72.23	CH_2	71.0
2	139.57	C	139.3
3[a]	129.77	CH	128.6
6[b]	130.84	CH	128.9
5	129.19	CH	128.2
6[b]	130.84	CH	128.9
7[a]	129.77	CH	128.6
Glucose	moiety		
G1	103.80	CH	103.3
G2	75.64	CH	75.0
G3	78.58	CH	78.1
G4	72.19	CH	71.9
G5	78.53	CH	77.5
G6	63.31	CH_2	63.1

* Chemical shifts were assigned on the basis of a Jmod experiment and by comparison with published reference data: **A**, benzyl β-D-glucopyranoside (*31*).
[a-b] interchangable values.

The identified compound **4** is a known natural constituent of various sources, as found e.g. in *Vitis vinifera* grapes (*6*), *Carica papaya* fruits (*31*), sour cherries (*7*), strawberries (*11*) and fresh tea leaves (*32*). This is the first report about the occurrence of the glucoside **4** in tomatoes.

2-Phenylethyl β-D-gentiobioside. The hydrolysis screening (β-glucosidase and hesperidinase) of the fractions obtained by a HSCCC separation of a more polar fraction led to a conjugate of 2-phenylethanol (**5a**) in the earlier part of the chromatogram (fig. 5). The analysis by DCI mass spectrometry with ammonia showed a strong ion at m/z 464 with an relative abundance of 62% representing the pseudo-molecular ion $[M+NH_4^+]$. The molecular ion at m/z 446 was found with a relative abundance of 2 %. The ^1H NMR spectrum exhibited two doublets at δ 4.31 (J=7.8 Hz) and 4.37 (J=7.8 Hz) for two anomeric protons, indicating the presence of two β-glycosidic linkages. Based on this data, the presence of a conjugate of 2-phenylethanol and two hexose sugars could be assumed. The ^1H NMR and ^{13}C NMR data (Table V and Table VI) obtained for compound **5** showed a strong coincidence with the data published for the aglycone moiety of 2-phenylethyl β-D-glucoside (*31*). The ^{13}C NMR data for the sugar moiety of compound **5** was fairly congruent with the data published for methyl β-D-gentiobioside (*33*). The Jmod experiment showed a downfield shift of about 7 ppm in the resonance of the carbon atom at position G-6. This effect is also known as glycosylation shift (*34*) and gives further evidence for a 1 →6 linkage between the two glucose molecules. Moreover we also compared the ^1H and ^{13}C NMR data of compound **5** to amygdalin (mandelonitrile β-D-glucopyranosyl (1→6)-β-D-glucopyranoside), prunasin (D-mandelonitrile β-D-glucoside) as well as gentiobiose in order to establish the identity of the sugar moiety.



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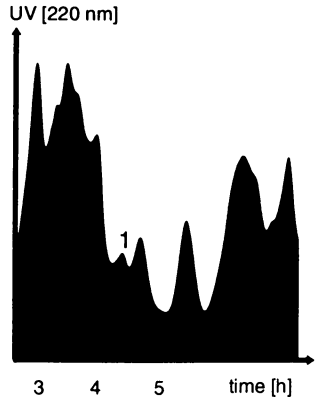

Figure 4. HSCCC separation of benzyl β-D-glucopyranoside. Conditions: Sample, 200 mg; solvent system, 2-pentanol/water (1:1); mode, "head to tail" (lower phase = mobile phase); stationary phase content, 70 %; flow, 1 mL/min; pressure: 80 psi.

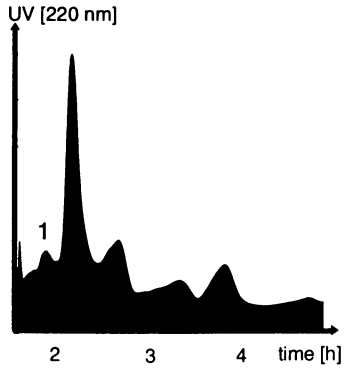

Figure 5. HSCCC separation of 2-phenylethyl β-D-gentiobioside. Conditions: Sample, 84 mg; solvent system, 2-pentanol/water (1:1); mode, "head to tail" (lower phase = mobile phase); stationary phase content, 70 %; flow, 1 mL/min; pressure: 80 psi.

Table V. ^1H NMR spectral data of the isolated 2-phenylethyl β-D-gentiobioside (5) and of reference compounds (δ relative to TMS, 400 MHz, CD_3OD)

Isolated	gentiobioside (5)		A
Aglycone	moiety		
δ (ppm)	Signal	Assignment	δ (ppm)
2.93	2H, br. t (7)	$H_{a/b}$-2	2.91
4.07	2H, m	$H_{a/b}$-1	4.05
7.26	5H, m	H-4, H-5, H-6, H-7, H-8	7.23
Sugar	moiety		B
3.2	2H, m	H-2; H-2'	3.25
3.3-3.44	6H, m	H-G3; H-G3'; H-G4; H-G4'; H-G5; H-G5'	3.22 - 3.46
3.65	1H, dd (12; 5)	H-$G6_b$'	3.65
3.77	1H, m	H-$G6_b$	3.82
3.85	1H, dd (12; 2)	H-$G6_a$'	3.88
4.14	1H, dd (12; 2)	H-$G6_a$	4.20
4.31[a]	1H, d (7.8)	H-G1'	4.35
4.37[a]	1H, d (7.8)	H-G1	4.55

Coupling constants (J in Hz) in parentheses.

Assignment were done by the aid of published data (*35*) and own reference compounds: **A**, 2-phenylethyl β-D-glucopyranoside (*31*); **B**, sugar moiety of amygdalin (mandelonitrile β-D-glucopyranosyl (1→6)-β-D-glucopyranoside; Sigma Chemical Comp.).

[a] Interchangeable values.

Table VI. ^{13}C NMR spectral data (100 MHz, CD_3OD) of the isolated 2-phenylethyl β-D-gentiobioside (**5**) and of reference compounds

Isolated glucoside			**A**
Aglycone	moiety		
Position	δ* (ppm)	Jmod	δ* (ppm)
1	71.90	CH_2	70.4
2	37.23	CH_2	36.4
3	140.05	C	139.4
4[a]	130.05	CH	129.3
5[b]	129.37	CH	128.5
6	127.21	CH	126.4
7[b]	129.37	CH	128.5
8[a]	130.05	CH	129.3
Sugar	moiety		**B**
G1	104.84	CH	104.5
G2[c]	75.08	CH	74.0
G3	77.93	CH	77.0[d]
G4	71.57	CH	71.2
G5	77.04	CH	76.1
G6	69.75	CH_2	70.0
G1'	104.43	CH	104.0
G2'[c]	75.04	CH	74.0
G3'	77.99	CH	77.2
G4'	71.40	CH	71.0
G5'	78.02	CH	77.2
G6'	62.73	CH_2	62.5

* Chemical shifts were assigned on the basis of a Jmod experiment and by comparison with published reference data as well as reference compounds: **A**, aglycone moiety of 2-phenylethyl β-D-glucopyranoside (*31*); **B**, methyl β-gentiobioside (*33*).

[a-c] interchangable values.

[d] According to literature and own data the published value for 71.0 ppm (*33*) has been corrected to 77.0 ppm.

In earlier studies the aglycone 2-phenylethanol was found as a major component in the bound flavor of tomatoes (*3*, *4*). The sugar moiety β-D-gentiobiose [β-D-glucopyranosyl (1→6)-β-D-glucopyranose] is well known for cyanogenic glycosides, such as amygdalin and derivatives (*35*) or linustatin and neolinustatin (*36*). Recently Winterhalter and coworkers (*37*) isolated and characterized the β-D-gentiobioside of 3-hydroxy-β-ionol from quince fruit (*Cydonia oblonga*). In tomatoes p-coumaryl-1-gentiobiose has been found by the group of El Khatib (*38*). This is the first report of compound **5** as a constituent of tomatoes.

CONCLUSIONS

The application of 3 different countercurrent chromatography systems to the separation of polar glycoconjugates from tomatoes emphasized significant advantages of CCC such as sample capacity and versatiliy. Although it is difficult to obtain absolutely pure samples by an exclusive use of CCC-techniques it is possible to reach a very high level of purity by optimization of the applied solvent system.

Using preparative CCC-techniques enabled the isolation of 5 glycoconjugtes from tomatoes. By means of ^{1}H and ^{13}C NMR spectroscopy as well as mass spectrometry the structure of adenosine (**1**), p-coumaric acid β-D-glucopyranoside (**2**), 3-(4-hydroxyphenyl)propionic acid β-D-glucopyranoside (**3**), benzyl β-D-glucopyranoside (**4**) and 2-phenylethyl β-D-gentiobioside (**5**) have been determined. While the glycoconjugates **1** and **2** were previously identified constituents of tomatoes, the glucosides **3** and **4**, as well as the gentiobioside **5** have been characterized in tomatoes for the first time.

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Reference to a company and/or product named by the Department is only for the purpose of information and does not imply approval or recommendation of the product to the exclusion of others which may be suitable.

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Chapter 16

Free and Bound Volatile Components of Temperate and Tropical Fruits

D. Chassagne and J. Crouzet

Unité de Microbiologie et Biochimie Industrielles, Institut des Sciences de l'Ingenieur, Université de Montpellier II, 34095 Montpellier Cédex 05, France

The distillation extraction and dynamic headspace study of free volatile components present in temperate (apricot) and tropical (mango) fruit cultivars revealed important qualitative and quantitative differences. Terpenic alcohols, linalool, α-terpineol, nerol, nerol derivatives, and linalool oxides, are partially responsible for the floral and fruity flavor of apricot. They are most important in aromatic cultivars. In mango, major differences were observed in terpenic hydrocarbons. Increased concentrations of terpenic hydrocarbons from heat treatment of the purees was considered as indicative of the presence of glucosidically bound components. These non volatile derivatives were found to be 4 to 5 fold more abundant than free volatile compounds in aromatic culivars. Apricot glucosidically bound components, isolated using several chromatographic methods, were identified using HPLC and soft ionization tandem MS. In some cases partial hydrolysis of the saccharide moiety was used for identification. Glucosides were the major glucosidically bound components in apricot and Mango. Glucosides, arabinoglucosides, rutinosides and gentiobiosides represent about 80% of bound compounds in passion fruit. Cyclodextrin bounded stationary phase was employed to separate the diasteroisomers of linalool and α-terpineol in apricot, grape and passion fruit.

It is generally recognized that fruit aroma varies qualitative and quantitatively depending on the cultivar, maturity stage, climatic and cultural conditions and the production area for each cultivar. Differences in free and bound compounds from temperate fruits such as apricot as well as tropical fruits such as mango or passion fruits have been reported. In the case of apricot, lactones, identified as being responsible for the background aroma are more important in the cultivars,

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Polonais and Rouge du Roussillon (1). On the other hand, terpenic alcohols are also considered as contributors of the fruity aroma of apricot (1,2).

Lactones, esters and fatty acids are important contributors of mango aroma. However, monoterpene and sesquiterpene compounds responsible of the tropical, turpentine like aroma of this fruit represent 60 to 90 % of total mango volatiles. Important differences concerning the nature and the concentration of these compounds have been pointed out.

More recently glycosidically bound volatile compounds, first detected in flowers or in grapes, were identified in several fruits, specifically in; apricot (3,4), mango (5,6) and passion fruit (7,8). The reported results were generally obtained from different cultivars produced in different places, and in some cases the identification of the cultivars was not known.

The aim of the present report is the study of free and bound volatile compounds of several specific apricot and mango cultivars all grown at the same location. At the same time, preliminary results concerning passion fruits of different origins are also given.

Free volatile compounds.

Apricot Rouge du Roussillon, a very aromatic cultivar grown in the South of France has been extensively studied in our laboratory (2, 9-13). The free volatile compounds were isolated by vacuum distillation and fractionated using silica gel chromatography. The sniffing of the isolated fractions and of the extract obtained after trapping the head-space on Chromosorb 105 indicates that terpinene-4-ol, α-terpineol, nerol geraniol and perhaps 2-phenylethanol are primarily responsible for the fruity aroma of this cultivar.

Table I. Terpenic alcohols identified from several apricot cultivars (mg/kg)

	Cultivars			
Compounds	Polonais	Bergeron	Précoce de Tyrinthe	Rouge du Roussillon
cis linalool oxide	0.2	0.3	0.2	0.3
trans linalool oxide	0.8	0.7	0.4	1.0
linalool	13.2	3.8	1.5	9.8
4-terpineol	0.3	0.1	0.2	0.3
α-terpineol	0.5	0.2	0.2	0.5
geraniol	0.1	0.1	0.1	0.1
nerol	0.2	0.07	0.1	0.4
total	15.3	5.3	2.7	12.4

The quantities of terpenic alcohols isolated from several apricot cultivars apricot obtained from the INRA orchard (Manduel France) using dynamic head-space trapping on charcoal-graphite with microwaves desorption are given in Table I. Highest concentrations of total terpenic alcohols were found in highly aromatic cultivars such as Polonais (15.3 mg/kg) and Rouge du Roussillon (12.4 mg/kg) and

lowest in less aromatic cultivars such as Bergeron (5.3 mg/kg), or Precoce de Tyrinthe (2.7 mg/kg).

As shown in Table II, major differences in terpene hydrocarbons and terpene alcohol concentrations in the mango cultivar extracts obtained by SDE. The fruit had been obtained from an orchard located near Dakar (Senegal). As shown in Figure 1, these differences are clearly noticeable in the Chromatograms from extracts obtained from the African mango and Papaya cultivars using the dynamic head-space technique.

Table II. Terpenic compounds identified from several mango cultivars

	cultivars					
compound	mango	Governor	Peach	Papaya	Muskat	Amelie
Hydrocarbons (mg/kg)	6.8	3.1	1.1	2.4	1.2	1.3
(% of Volatiles)	49	60	20	44	47	15
Alcohols (mg/kg)	0.35	0.14	0.31	0.21	0.05	0.81
(% of Volatiles)	2.5	2.4	5.7	3.8	1.9	9.4

The increase of some terpenic compound concentrations observed during heat treatment of mango or apricot puree (10,14) was considered as indicative of the presence of glycosidically bound volatile components in these fruits. More particularly the dramatic increase of α-terpineol during heat treatment of the mango puree cannot be explained by rearrangement reactions of terpenic compounds. The acidic hydrolysis of glycoside derivatives of this alcohol at the pH of the puree (3.9) was considered as more probable.

Characterization of glycosidically bound components

The presence of glycosidically bound volatile components was established using the rapid analytical technique described by Dimitriadis and Williams (15). Bound compounds are present in all the mango varieties studied, but they are in especially high concentrations in the ungrafted African mango and to a lesser degree in Governor cultivar (6). For the African mango the value obtained for the bound compounds and the ratio between the bound and free forms are comparable to those found for aromatic grape cultivars (15).

Shown in Table III are the relative concentrations of free and bound volatile compounds, along with the ratio of bound/free volatiles in several apricot cultivars. Similar results were obtained for passion fruit of different origins (16). The aromatic cultivar, Rouge du Roussillon, contained the highest levels of bound compounds as well as the highest bound/free ratio (5.2). The high bound/free observed for the less aromatic cultivar, Precoce de Tyrinthe, may be attributed to the very low value of free compounds.

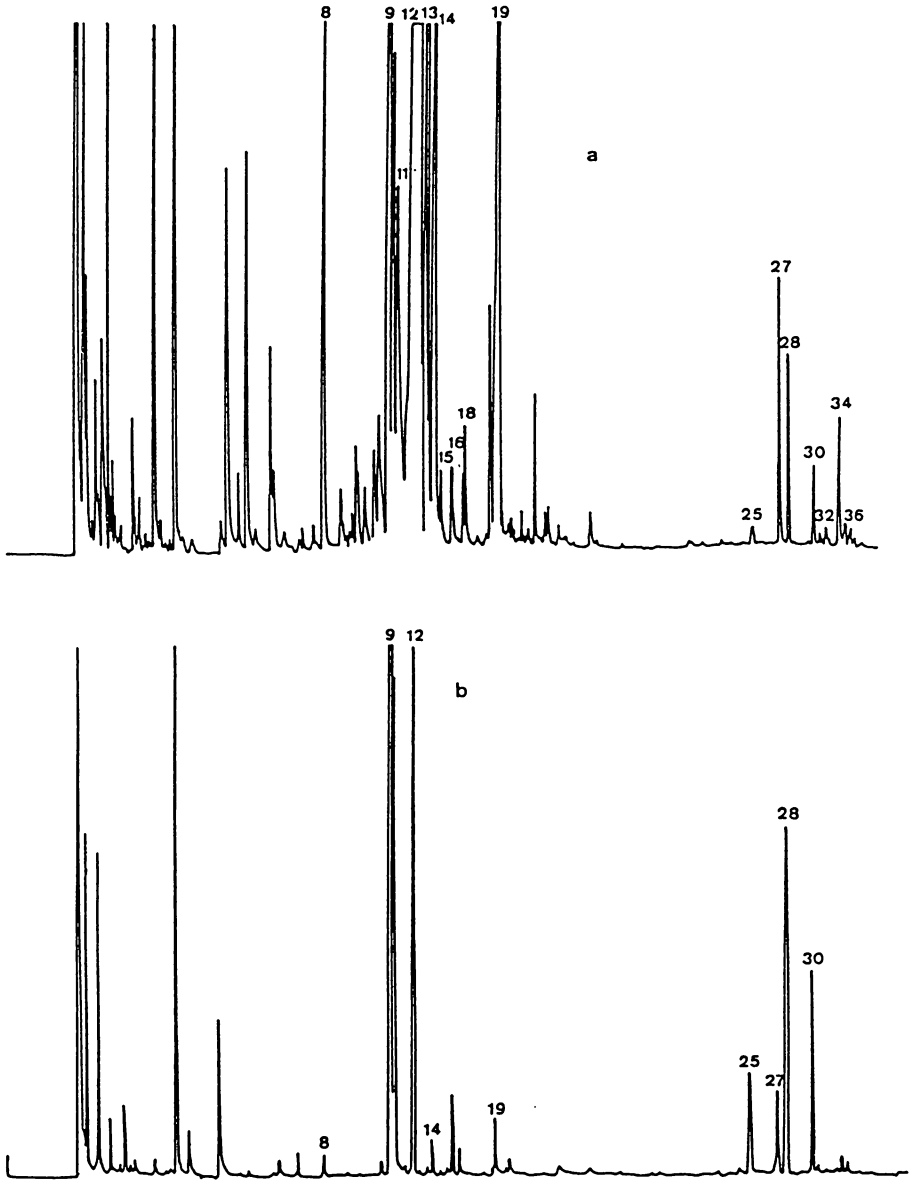

Fig. 1 Gas chromatogram of aroma compounds isolated by adsorption on activated charcoal-graphite trap and microwaves desorption from a) african mango, b) Peach cv. J &W 30m x 0.25 mm (i.d.) DB 5 WCOT capillary column. The temperature program was 10 min isothermal at 60°C and then from 60 to 250°C at 4°C /min.

Table III. Free and bound volatile compounds present in several apricot cultivars

cultivar	free compounds	bound compounds	bound/free
Bergeron	1.9	3.2	1.6
Canino	2.8	3.6	1.3
Rouge du Rousillon	1.5	7.6	5.2
Precoce de Tyrinthe	0.6	3.1	5.4

Important values for bound compounds were found for purple passion fruit of different origins and more particularly in fruits originated from Zimbabwe and Burundi (16).

Study of glycosidically bound volatile components

The glycosidic fractions of apricot (Rouge du Roussillon), mango (African mango) and passion fruit (from Zimbabwe) were isolated by adsorption on C 18 reversed-phase (17) or Amberlite XAD2 (18).

Total Hydrolysis. The first step of the study of glycosidically bound components is the identification of the sugar moiety and the volatile compounds released by acidic or enzymatic hydrolysis. The enzymatic method being preferred for the study of the aglycone moiety because of the risks of rearrangement of the terpenes during acidic hydrolysis. In these studies glucose, arabinose and rhamnose were detected by TLC after acid hydrolysis of the terosidic pool of the three fruits.

The volatile compounds were identified after enzymatic hydrolysis (hemicellulase REG 2) of the heterosidic pool. The results obtained for passion fruit are given table IV (19). These results are in good agreement, particularly concerning the C_{13} isoprenoids with those reported by Winterhalter (8). The presence of mandelonitrile and benzaldehyde produced by the enzymatic hydrolysis of prunasin and/or sambunigrin (20) should be noted. According to these authors, hydrogen cyanide is released by enzymatic hydrolysis of the cyanogen by emulsin.

Mass spectrometry studies. Apricot glycosidic compounds were separated by silica-gel chromatography, gel filtration on Fractogel TSK HW-40 S and preparative over-pressure layer chromatography (OPLC) (12). The use of chemical ionization in negative mode with ammonia as reagent gas (NICI) for the structural study of the isolated fractions indicated that glucosides, $(M-H)^-$ m/z 263 (hexyl), 269 (2-phenylethyl), 315(terpene alcohols), 331(linalool oxides or dienediols) were largely present in the fruit. Some arabinoglucosides of monoterpene alcohol (m/z 447) and of linalool oxides or dienediols (m/z 463) were also detected.

Table IV. Volatile compounds identified in passion fruit pulp by GC and GC-MS after enzymatic hydrolysis (hemicellulase REG 2) of the heterosidic pool

(Z)-3-hexenol	isoeugenol
1-hexanol	unidentified norisoprenoid
benzaldehyde	hydroxydihydronaphtalene
benzyl alcohol	unidentified norisoprenoid
1-octanol	unidentified norisoprenoid (B)
4-nonanol	4-hydroxy-ß-ionol
linalool	3-oxo-α-ionol
2-phenylethanol	4-oxo-ß-ionol
4-ethylphenol	unidentified norisoprenoid (C)
α-terpineol	unidentified norisoprenoid (D)
nerol	3-oxoretro-α-ionol (1)
geraniol	4-oxoretro-7,8-dihydro-ß-ionol
4-allyphenol	3-oxoretro-α-ionol (2)
mandelonitrile	dehydrovomifoliol
eugenol	vomifoliol

However it was not possible to distinguish between aglycone isomers. For example each terpene alcohol isomer produces a peak at m/z 315. Tentative identification may be achieved using low energy collisionally activated (CAD) fragmentation patterns in NICI tandem mass spectrometry (MS/MS). It was shown (21) that the relative abundance of the ionic species detected in the spectrum was dependent on the nature of the aglycone moiety. Fragmentation rules were established from several synthetic glycosides. The application of the fragmentation rules to the spectra obtained from the OPLC fractions allowed us to tentatively identify; benzyl, 2-phenylethyl, linalyl, α-terpinyl, neryl and geranly glucosides. Linaly and α-terpinyl arabinoglucosides were also tentatively identified.

On the other hand, the use of ND_3 as reagent gas in NICI allows the differentiation of isomers such as dienediol and linalool oxide glucosides which both possess a parent ion at m/z 331 and very similar low energy CAD spectra. In the presence of ND_3, the parent ion is shifted to m/z 334 for linalool oxide glucosides possessing four acidic protons whereas for dienediol glucosides (five acidic protons) a shift of four mass unit is found. Four linalool oxides and four dienediol glucosides were tentatively identified (Table V). In the same way a dienediol arabinoglucoside, (M-H)- m/z 463 shifted to m/z 469 (Md7-D)- was detected.

Identification by HPLC. HPLC on a C_{18} reversed-phase was used for the identification of glucosides when authentic samples are available: linalyl, α-terpinyl, neryl, geranyl, 2-phenylethyl, benzyl and hexyl glucosides and rutinosides (12). Linalyl and α-terpinyl arabinoglucosides were identified after sequential hydrolysis of the glycoside (22). After the action of an arabinase isolated from *Aspergillus niger* pectinase Reyne et al. (23), observed a decrease in the peak corresponding to the glycoside and the appearance of a peak with the same retention time as the corresponding glucoside.

Table V. Apricot (cv. Rouge du Roussillon) bound glucosidic compounds identified by NICI mass spectrometry using NH_3 and ND_3 as reagent gas

parent ions				number of
$(M-H)^-$	$(M-D)^+$	fragmentation	suggested structure	compounds
331	334	2M	linalyl oxide glucoside	4
331	335	2M	dienediol glucoside	2
331	335	1M	dienediol glucoside	2
463	469	-	dienediol arabino glucoside	1

These results, are in good agreement with the preliminary report of Salles et al. (11). They demonstrated that about 90% of the glycosidically bound volatile compounds in apricot were glucosides. The remaining 10%, are disaccharidic derivatives. However, in this group, only arabinoglucosides were detected. Dissacharidic compounds are predominant in other fruits such as aromatic grapes. Between 32 to 58 % arabinosylglucosides, 28 to 46% apiosylglucosides, 6 to 13 % rhamnosylglucosides (rutinosides) and only 4 to 9 % glucosides have been reported by Bayonove et al. (24). Preliminary results obtained for glycosidically bound volatile compounds in passion fruit reveal that there are about 22% glucosides, 12% arabinosylglucosides, 39 % rutinosides and 27 % gentiobiosides present (19). Among these compounds, 2-phenylethyl, linalyl, α-terpinyl, geranyl and neryl glucosides and rutinosides and benzyl alcohol rutinoside have been identified by analytical OPLC and HPLC from the fractions isolated by preparative OPLC.

When a cyclodextrin bounded-phase (Cyclobond I) was used, the two diastereoisomers resulting of the binding of tertiary monoterpene alcohols to the glucoside unit are separated (13).

These results show that linalool is bound as a glucoside, arabinoglucoside or rutinoside and is present at 85 % as (S)-(+) isomer in apricot and passion fruit. More than 95 % of glycosidically bound linalool in grapes, is found as the (S) (+) isomer. In apricot, 70 % of the (S)-(-)isomer and 30% of the (R)-(+) isomer of α-terpineol are bound to a glucose unit.

Conclusion

Important qualitative and quantitative differences were found for free terpenic compounds present in temperate (apricot) and tropical (mango) fruits according to the nature of the cultivar for the same origin. The glycosidically bound volatile compounds are about 4 to 5 fold more abundant than free compounds in aromatic cultivars.

The determination of the structure of isolated heterosidic fractions by MS, MS-MS and HPLC shows that glucosides are the major glycosidically bound components in apricot (90%) whereas in passion fruit these compounds represent only 22% of the bound compounds.

The ratio of the two diastereoisomers of linalyl and α-terpenyl glycosides present in apricot passion fruit or grapes varies according to the nature of the fruit.

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Chapter 17

Developments in the Isolation and Characterization of β-Damascenone Precursors from Apples

Deborah D. Roberts and Terry E. Acree

Department of Food Science and Technology, New York Agricultural Experiment Station, Cornell University, Geneva, NY 14456

β–Damascenone is a potent aroma compound in a variety of natural products, with a low odor threshold of 2 pg/g in water. Its characterizing presence in heated apple products is due to liberation from precursors. The isolation of β–damascenone precursors from apples *Malus domestica*, Borkh. CV Empire was performed using Amberlite XAD-2 and C-18 flash chromatography followed by C-18 HPLC. At least eight separate precursors were found, and the most abundant was further characterized. Ion spray tandem mass spectrometry with its mild ionization conditions provided molecular weight and structural information. Enzymatic hydrolysis-GC/MS and trimethylsilation-GC/MS gave aglycone and sugar information on the precursor. The most abundant precursor, present at 4.6 ng/g apple (expressed as amount of β–damascenone liberated under acid hydrolysis), was the 9(or 3)-α-L-arabinofuranosyl-(1,6)-β–D-glucopyranoside of the acetylenic diol. The second most abundant precursor, present at 3.1 ng/g apple, is a more polar glycoside of the acetylenic diol. Investigations into grasshopper ketone as a β-damascenone precursor found that while it may be a biosynthetic precursor, it is not a direct precursor, producing β-damascenone under acid hydrolysis conditions with only a 0.006% yield. A mechanistic pathway for the conversion of the acetylenic diol to β–damascenone was proposed.

β–Damascenone is an unusually potent aroma compound, with a threshold of 2 pg/g in water [*1*]. It has a fruity odor and has been found in a variety of natural products. A sensory effect from β–damascenone in these products is assured because of its low threshold.

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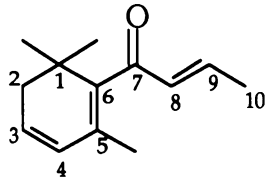
β–Damascenone's importance to the flavor of over 40 cultivars of apples was demonstrated using gas chromatography olfactometry (GCO) a bioassay technique that uses humans to sniff the effluent of a gas chromatograph for odor activity. In a GCO process called CharmAnalysis [*2, 3*] serial dilutions (3 fold) of Freon-113™ extracts



β–Damascenone

from apple cultivars were separated using capillary gas chromatography and sniffed using a high resolution olfactometer [*4*]. During the GCO analysis, the subject indicated when an odor was detected, repeating the runs with more dilute samples until no odors were detected. Compounds detected after many dilutions had high odor potency (dilution value) and contributed substantially to the aroma. A charm chromatogram was made for each apple cultivar by plotting dilution value verses retention indices. After averaging the charm chromatograms over the 40 cultivars, β–damascenone was the most potent odor detected.

β–Damascenone has been found to increase substantially when several natural products are heated. In the process of making tomato paste, macerated tomatoes are run down metal towers with heat and reduced pressure where most volatiles are lost by steam volatilization. β–Damascenone's concentration, however, increased from 1 ng/g in the fresh tomato to 14 ng/g in the paste [*1*]. When Satsuma mandarin juice was heated to 80°C at 10°C/min and then cooled, β–damascenone was detected by GC/MS with an odor described as that of overripe fruit. In the unheated juice, β–damascenone was not found by GC/MS [*5*]. Apples also showed an increase in β–damascenone concentration with post-harvest processing such as heating. GCO was performed on heated and unheated Empire apple juice [*6*]. β–Damascenone contributed 32% of the total aroma potency of heated apple juice yet only 1.6% in fresh apple juice. Likewise, in the production of apple brandy from Jonathan apples, β–damascenone was not found in the unprocessed apple or apple mash held at 11°C but was found in apple brandy distilled at 79-90°C at 198 mg/L [*7*]. β–Damascenone's formation from precursor compounds upon heating is evident. In processed apple products, β–damascenone is the most potent and perhaps the most important odor-active volatile.

Chemistry of β–damascenone precursors

β–Damascenone is a C-13 nor-isoprenoid compound which is believed to be derived from xanthophylls. Neoxanthin, the principal leaf xanthophyll in green leaves [*8*], decreases during berry ripening [*9*]. As shown in Figure 1, neoxanthin is hypothesized to oxidize to grasshopper ketone (3,5-dihydroxymetastigma-6,7-dien-9-one) [*10*] by enzymatic cleavage of the 9, 10 double bond [*11*]. Grasshopper ketone is so called because it was first isolated from the ant-repellent secretions of the grasshopper *Romalea microptera* [*12*]. The compound grasshopper ketone was also detected in Riesling wine as well as in other grapes [*13*].

While most C-13 nor-isoprenoids are oxygenated at C-9, β–damascenone bears a C-7 oxygen. Allenic or acetylenic intermediates would allow the transposition of oxygen from C-9 to C-7. Several such compounds (**a**, **b**, **c** in Figure 2) have been shown to produce β–damascenone after treatment. A synthesized acetylenic triol **b**

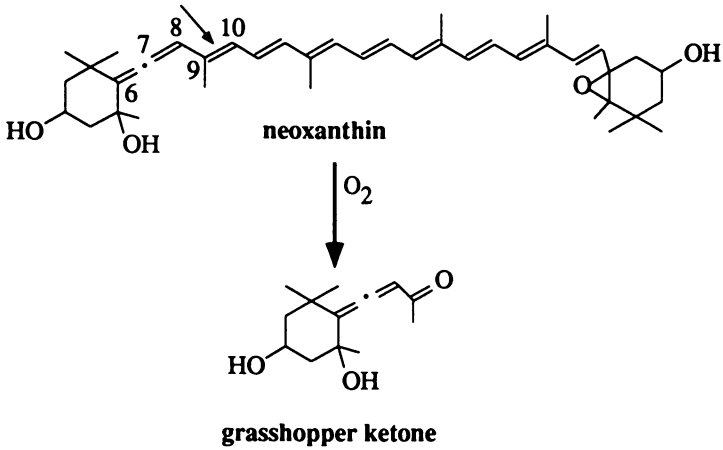


Figure 1. Proposed enzymatic conversion of neoxanthin to grasshopper ketone.

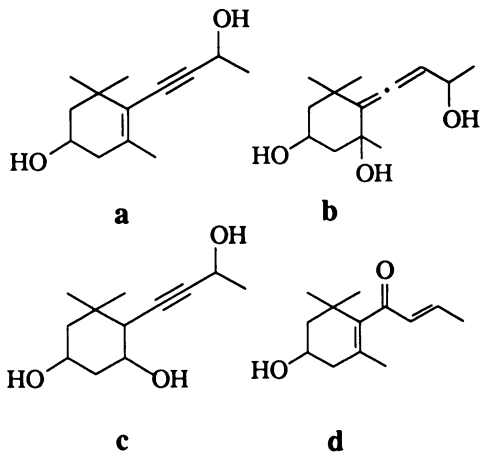


Figure 2. Allenic and acetylenic compounds (**a**, **b**, **c**) are β-damascenone precursors with 3-hydroxy-β-damascone **d** as the major product of the reactions.

produced β-damascenone upon treatment with sulfuric acid, with a proposed dehydration into the acetylenic diol first [*14*]. The relationship between the acetylenic diol **a**, acetylenic triol **b**, allenic triol **c** and β–damascenone was further investigated using synthesized compounds under various heating conditions [*15, 16*]. All (**a**, **b**, **c**) three produced β–damascenone, although there were variations in the amounts depending on the conditions. 3-hydroxy-β–damascone **d** was the major product of the reactions. 3-hydroxy-β–damascone, itself, has been tested as a precursor with negative results [*10*].

Natural product isolations have generated evidence for multiple β–damascenone precursors. Riesling wine's precursor fraction was isolated using a C-18 column and then subject to DCCC (droplet counter-current chromatography), resolving at least three β–damascenone precursors [*17*]. Additional evidence for multiple β–damascenone precursors was seen in the skin of Concord grapes [*18*]. Analytical TLC and HPLC separation showed the presence of multiple precursors with chromatographic properties similar to methyl mannoside and ocyl glucoside.

Elucidation of the identity of β–damascenone precursors has several important consequences. First, it would allow the direct measurement of a fruit's potential to develop the intense characteristic floral odor of β–damascenone. Particularly in fruits that will be heated, this would be the best predictor of its final β–damascenone aroma intensity. This information may be used by breeders or food processors in selecting the optimal variety, vineyard, or farm. Second, information about the chemical properties of the precursors such as their susceptibility to enzymatic hydrolysis or behavior under different growth and processing conditions could be obtained. Third, the activity of the precursors such as migration in the plant or the mechanism of conversion to β–damascenone could be determined to increase understanding of the biosynthetic pathway. The research described here begins the investigation by determining the number of β–damascenone precursors present in apples and characterizing the most abundant. Figure 3 shows the general isolation and characterization methodology. The details can be found in Roberts, *et al.*, 1994 [*19*].

Detection of eight β–damascenone precursors

Eight unique precursors were separated by C-18 HPLC using a gradient of 10 to 100 % methanol in water. Rechromatography of the original 11 fractions containing precursors determined that at least eight were unique. The relative sizes of the precursors were estimated by gel permeation chromatography and found at least 2 triglycosides, 4 diglycosides, and 2 polyols [*19*]. Table I shows the % methanol elution of the eight precursors.

The amounts present in apples were calculated as ng/g ß-damascenone equivalents after acid hydrolysis. In total, β–damascenone precursors were present at a level of 18 ng/g apple, with the most abundant precursor at about 4.6 ng/g apple. Liberation of the precursors would generate a β–damascenone level of 9000 times its odor threshold (2 pg/g) or 9000 odor units. Thus, although 3 ng/g is a very low amount for chemical characterization, it produces a large sensory response in heated apple products.

Use of ion spray mass spectrometry

Ion spray mass spectrometry is especially suited for the analysis of thermally labile flavor compounds. Ion spray (or pneumatically assisted electrospray) is an API (atmospheric pressure ionization) interface for LC/MS. It uses nitrogen gas to nebulize a liquid stream eluting from a charged capillary. Gas-phase ions are formed by ion evaporation from

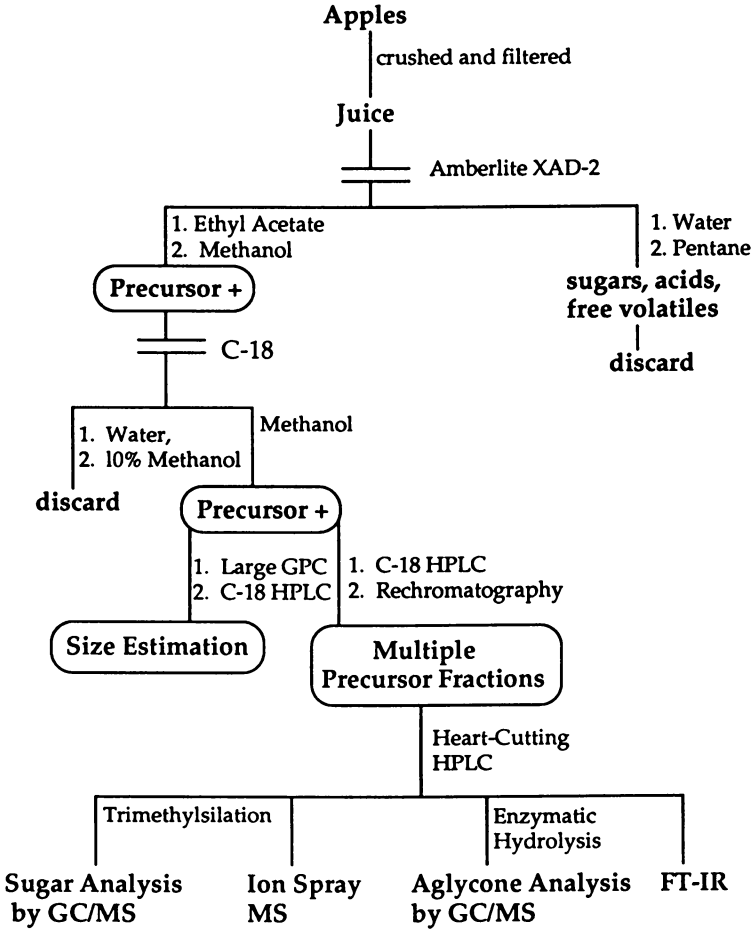


Figure 3. Isolation and characterization scheme of β-damascenone precursors from apples.

highly charged condensed phase droplets [*20*]. This soft ionization technique allows non-volatile molecules to be analyzed without thermal degredation, which could occur with a thermospray interface [*21*]. Allen *et al.* have published a good review of ion spray applications [*22*].

Table I. Amount and HPLC elution parameters of β-damascenone precursors

β-Damascenone Precursor	% Methanol in Water C-18 HPLC Elution	Amount in Apples (ng/g)*
1	39.6	2.2
2	42.2	3.1
3	44.7	1.5
4	49.9	2.0
5	52.4	1.9
6	55.0	4.6
7	67.9	1.0
8	73.0	1.5
Free β-Damascenone	88.4	< 1.0

* measured as β-damascenone equivalent amount

Mass spectral analysis of complex mixtures, as are often the case in natural products can be accomplished using tandem quadrupole mass spectrometry. An instantaneous separation of compounds is achieved by the first mass analyzer (MS-1) while the second (MS-2), the electrostatic analyzer, produces the mass scale. Various modes allow the monitoring of fragment ions, precursor-ions, neutral-loss molecules, or multiple reaction monitoring [*22*]. The combination of ion spray LC/MS interface and tandem mass spectrometry provides a tool which will undoubtedly find great use in the analysis of natural products, especially flavor precursors whose application is demonstrated in this study.

The most abundant β–damascenone precursor **6** (Table I) was purified by HPLC and analyzed by ion spray tandem mass spectrometry. The spectrum is shown in Figure 4. Ammoniation of molecular ions results because of the presence of ammonium formate in the solvent. The ammoniated molecular ion is m/z 520 with a corresponding molecular weight of 502 ($C_{24}O_{11}H_{38}$). Glycosides fragment after protonation of the glycosidic oxygen by breaking the oxygen-sugar bond, transferring a hydrogen, and leaving behind a protonated alcohol [*23*]. This fragmentation mechanism produced m/z 209.

The ions produced by the ion spray mass spectrometry of precursor **6** are the glycosidic ammoniated precursor, m/z 520, and its daughter ions: protonated β–damascenone, m/z 191, and protonated acetylenic diol **a**, m/z 209. The presence of the ion m/z 209 (protonated acetylenic diol) from parent m/z 520 indicates that the molecule is a sugar adduct of the acetylenic diol. The acetylenic diol has been identified in tobacco [*24*], purple passion fruit [*25*] and in a free and unspecified bound form in wine and grapes [*15, 26*]. As discussed earlier, it is a known progenitor of β–damascenone.

Additional characterization by enzymatic hydrolysis and TMS (trimethylsilation)

Further information supporting the proposed precursor **6** structure was obtained by enzymatic hydrolysis - GC/MS and TMS - GC/MS. Enzymatic hydrolysis of precursor **6** and precursor **2** yielded the acetylenic diol **a** as determined by mass spectrum and retention time matches with an authentic standard [*19*]. The aglycone for precursor **6** and precursor **2** is the acetylenic diol **b**. Precursor **2** elutes from the HPLC at 42.2% methanol in water as opposed to 55% methanol in water for Precursor **6**. The more polar HPLC elution of Precursor **2** indicates that it may have more sugars attached or at different hydroxyl groups on the acetylenic diol.

TMS and GC/MS of precursor **6** determined which mono or disaccharides were attached. The hydrolyzed precursor produced monosaccharides which matched in retention time and mass spectra to arabinose and glucose authentic standards [*19*]. Additionally, the intact precursor showed arabinoglucoside which matched in mass spectrum and retention time to the α-arabinofuranosyl-α(β)-glucopyranose previously reported [*27*]. Although the mass spectra of arabinoglucoside and apioglucoside are very similar, the abundance of m/z 191 is a distinguishing feature [*27*]. A low abundance of ion 191 indicated that the disaccharide contained arabinose instead of apiose.

Tests with Grasshopper Ketone

An experiment with the grasshopper ketone was performed to determine if it could be a direct precursor to β–damascenone. A synthetic sample of grasshopper ketone, donated by the lab of C. H. Eugster from Universitat Zurich was heated in an aqueous solution at 90 C for 20 min at pH 2.3. The freon extract was analyzed for β-damascenone by GC/MS using an external standard curve. β-Damascenone yield from grasshopper ketone was 0.006 %. This compares to a yield of 5% β-damascenone from the allenic triol **b** and 2.8 % from the acetylenic diol **a** under similar conditions [*15-16*]. Thus, although the grasshopper ketone may be a biosynthetic precursor to β-damascenone in the apple, it is not a direct chemical precursor.

Acetylenic Diol, a β–damascenone precursor

Precursor **6** has been identified as the 9(or 3)-α-L-arabinofuranosyl-(1,6)-β–D-glucopyranoside of the acetylenic diol. The 9-form is shown in Figure 4. This identification prompted a mechanistic hypothesis of the acetylenic diol to β–damascenone conversion pathway, shown in Figure 5. An acidic environment is proposed because the conversions of the acetylenic diol to β–damascenone occur at low pH. 3-Hydroxy-β–damascenone **d** is the major product when the acetylenic diol is heated at low pH [*15*]. 3-Hydroxy-β–damascenone would be the major product if the reaction sequence in Figure 6 proceeded without the dehydration step 1. The product of step 2, megastigma-3,5-dien-7-yn-9-ol, was found when the allenic triol **c** was heated at pH 3 and hence proposed to be an intermediate in the acetylenic diol to β–damascenone conversion [*16*] . The reaction steps 3, 4, 5, and 6 comprise what is known as the Meyer-Schuster rearrangement. This rearrangement is the isomerization by a 1,3 shift, of secondary and tertiary α-acetylenic alcohols to α,β–unsaturated carbonyl compounds [*28*]. In steps 1 and 2, electrophilic attack by protons forms a secondary carbonium ion which expels a proton from an adjacent carbon. Electrophilic attack by protons (step 3) furnishes a carbonium ion which isomerizes to the allenyl cation (step 4). Hydration and enol to keto conversion comprise steps 5 and 6. The driving force for this reaction has been shown to be the irreversible processes which form unsaturated carbonyl

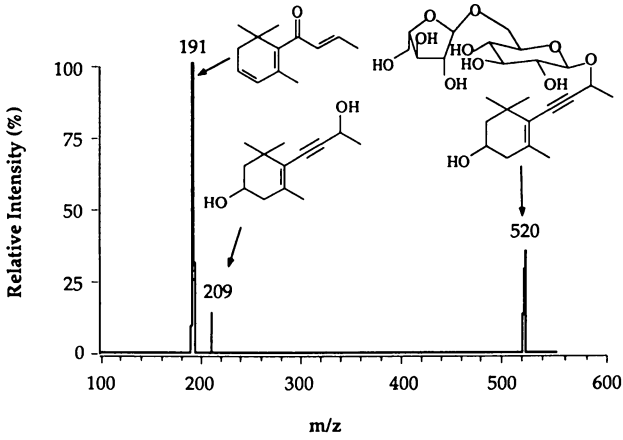


Figure 4. Ion spray mass spectrum of the most abundant precursor in apples (precursor **6**) showing the ammoniated molecular ion and protonated fragment ions.

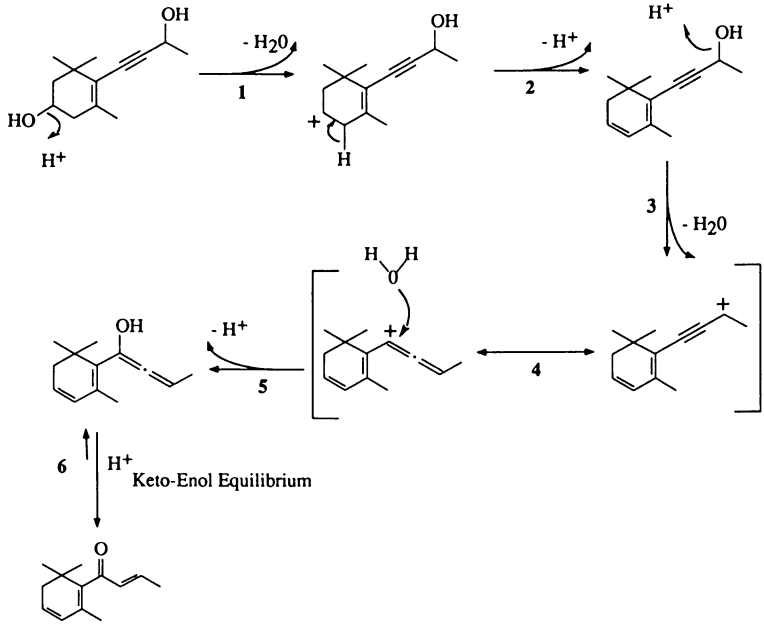


Figure 5. Proposed mechanistic pathway for the acid-catalyzed formation of β-damascenone from the acetylenic diol **a**.

compounds from the intermediate carbonium ions [*28*]. While this mechanism occurs with similar compounds in the Meyer-Schuster rearrangement, it has not been proven experimentally. Experiments which could further elucidate the pathway include the use of isotopic labelling, identifying the intermediates, and determining the K_{eq} of the keto-enol equilibrium.

Conclusion

The diverse nature of β–damascenone precursors was demonstrated by the identification of at least eight unique precursors. The most abundant was identified as the 9(or 3)-α-L-arabinofuranosyl-(1,6)-β-D-glucopyranoside of the acetylenic diol. The second most abundant is a more polar glycoside of the acetylenic diol. Elucidation of the relationships between the eight precursors and their formation pathways to β–damascenone would be a useful area of future research.

Acknowledgements

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Chapter 18

Flavor–Package Interaction

Assessing the Impact on Orange Juice Quality

G. Sadler[1], M. Parish[2], J. Davis[2], and D. Van Clief[2]

[1]National Center for Food Safety and Technology, 6502 South Archer Road, Summit-Argo, IL 60501
[2]Citrus Research and Education Center, University of Florida, 700 Experimental Station Road, Lake Alfred, FL 33850

Nitrogen-purged, orange juices (high oil, medium oil, low oil, and thermally abused) stored in glass with sterile strips of polymers (low density polyethylene, oriented polypropylene, nylon, polyethylene terephthalate, or ethylene vinyl alcohol co-polymer) did not suffer detectable sensory change during storage despite oil losses as great as 41%. However, d-limonene absorption greatly increased microbial proliferation in juice systems containing LDPE.

There has been a steady increase in the use of plastics in packaging since the 1960's. The tendency of aroma compounds to transfer between packages in close storage on store shelves was identified in the late 1960's (1). In the early 1980's, research speculated that volatile absorption by packaging materials might influence flavor intensity or alter characteristic flavor profile of foods packaged in plastic materials (2). In these studies, terpene hydrocarbons were observed to be absorbed at exceptionally high rates. This appeared to be especially important for citrus. Cold pressed citrus peel oils can contain over 95% d-limonene and smaller percentages of other terpene compounds. Citrus processors often add cold pressed peel oil to orange juice to optimize flavor. Early citrus flavor work identified d-limonene as an important component of citrus flavor. It therefore appeared likely that limonene absorption would be a good candidate for absorption-related flavor loss.

In reality, d-limonene and other unoxidized terpenes impart no significant flavor to citrus products (3,4). Early reports of d-limonene's importance to flavor can be traced to the former difficulty of removing active flavor compounds from d-limonene distillates. When d-limonene is present above its sensory threshold it is often considered a flavor defect (3).

Volatiles with polar functional groups (esters, carbonyl compounds, alcohols, etc) are more often associated with desirable citrus flavor. These compounds tend to

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be much less soluble in packaging materials than terpenes (5,6,7,8,9,10). A great deal of experimental sophistication has been used to study the interaction of these classes of compounds with a broad assortment of food-contact polymers. Although analytical methods are able to detect trivial levels of absorption they provide limited insight into the sensory consequences of volatile uptake.

Surprisingly little work has been devoted to understanding the sensory impact of volatile absorption. Early studies compared orange juice flavor differences between laminated paper/foil drink boxes and product stored in glass for at 25°C. Although researchers noted a significantly greater shelf life for the product in glass, they cautioned that oxygen exposure for product in juice boxes was much greater than for product stored in glass.(11) Therefore, the component of shelf life attributable to volatile absorption could not be distinguished from the portion of shelf life dictated by oxidation.

A recent study (12) indicated absorption of citrus aroma volatiles into a polyethylene/barrier laminated carton at 4°C did not significantly differ in sensory quality from their 4°C glass control. At this lower temperature, volatile absorption and oxygen permeation/reaction would be substantially slower than at 25°C. Improved flavor stability would be reasonably expected. Although the results of this study are likely solid, they are mitigated somewhat by the use of juice with mediocre initial sensory scores. This compresses the range of hedonic response available to sensory panelists.

The olfactory effect of volatile absorption has been analyzed using gas chromatography-olfactometry (4). Extracts of juices with and without prior polymer contact were subjected to GC separation. Peaks were analyzed by FID and human olfaction. Despite d-limonene losses of 70-80%, the odor intensity of polymer-treated juices were not perceptibly lower than polymer-free controls.

These studies suggest that volatile absorption, while instrumentally quantifiable, may not have significant sensory impact. This belief is also held by some citrus processors. Others feel volatile absorption may not be detectable in typical juices, but may be apparent in juices with very high or very low levels of peel oil. Juices with underlying flavor defects, such as oxidized or thermally abused flavor, have also been speculated to exhibit greater absorption-related flavor differences.

The effect of volatile absorption on quality attributes other than flavor have received little attention. The absorption of some flavors have been shown to increase the oxygen permeability of host polymers. This method has been exploited to determine the diffusion coefficient and relative magnitude of interaction in some polymer/flavor couplets (8). However, no effort has been made to determine whether this phenomenon significantly influences quality.

To date no work has examined the impact of volatile absorption on chemical and microbial properties of juice. However, anecdotal information among producers of unpasteurized juice holds that high peel oil juices have greater microbial stability than juices with typical peel oil. Dipentene, (a mixture of d-limonene and its l-isomer) has been used as an antimicrobial agent for many years. Treatment of citrus waste effluents and fermentation of citrus molasses are slowed by excessive levels of d-limonene. Therefore, it is not unreasonable to believe d-limonene may have some impact on the microbial environment in citrus juices. If d-limonene does influence microbial numbers then its removal through interaction with package materials may lead to shorter shelf life.

The purpose of this work was to determine whether absorption of flavor by 4 common packaging polymers, low density polyethylene (LDPE), polyethylene terephthalate (PET), nylon, or ethylene vinyl alcohol copolymer (EVOH) is sufficient to influence flavor of high oil, low oil, typical oil or thermally abused juice; and to determine if d-limonene absorption influences microbial numbers at concentrations typically found in commercial orange juice.

Experimental

Sensory Study

Characteristics of Orange Juice Used in Sensory Testing. High (0.023%) and low (0.009%) oil lots of orange concentrate were acquired through a major citrus processor. Lots were specifically selected for high flavor scores during preliminary in-plant sensory screening. Moderate oil (0.016%) juice was produced by blending high and low oil lots. All lots contained similar proprietary levels of citrus essence.

Juice Processing. Concentrates were reconstituted to 11.8°Brix with sterile distilled water. No subsequent thermal treatment was provided except for thermally abused samples which were prepared by recirculating reconstituted juice through a plate heat exchanger for 20 min. at 100°C.

Polymer Treatment. Polymer strips (2 x 10 cm) were cut from bulk rolls of LDPE, PET, nylon and EVOH. Nominal thickness for all films was 25 μ (1 mil) except for PET which was 23.4 μ (0.92 mil). A twist was introduced to the polymer strip which was stapled back on itself to form a kinked loop. The twist kept the strips from folding back upon themselves and limiting surface area available for absorption. Strips were sterilized in distilled water for 30 min. at 90°C. Strips of each material were aseptically transferred to milk dilution bottles containing 10 mL of 0.1% sterile peptone (Difco Laboratories, Detroit MI) broth. Duplicate aliquots (0.01 mL) were plated on orange serum agar (OSA) plates.

Juice Storage. Juice (1600 mL) was stored in a sterile 2 L erlenmeyer flasks with 1589 cm^2 of polymer. This represented 2 times the polymer surface to juice volume ratio found in standard gable-top containers (1.89 L). The headspace of the flasks were nitrogen purged to < 1% oxygen and capped with a saran-coated rubber stopper. Flasks were stored at 4.5 ± 0.5°C for 2 to 3 weeks. Flasks were agitated several times a week to allow all polymer surfaces access to flavor absorption. Controls were freee of polymer strips; but were otherwise subjected to all treatment and storage steps described above.

Juice Analysis, Chemical. Scott oil values were determine on all juices at the end of storage using analytical methods standard to the citrus industry (13).

Juice Analysis, Sensory. Triangle taste panel studies examined sensory differences between polymer-treated juices and polymer-free controls. Fifteen to twenty-two

experienced panelists were offered samples in random presentation. If a difference was perceived a statement of preference was requested. Triangle data was evaluated using tables of Roessler et al.(14). Samples were considered significantly different if they differed at the $P \leq 0.05$ level of significance.

Microbial Study

Characteristics of Orange Juice Used in Microbial Study. D-limonene-free (pumpout) 72°Brix low pulp concentrate was diluted to 50 °Brix with sterile distilled water. Various levels of 99+% d-limonene (Tastemakers, Lakeland, FL) were added which would result in single strength peel oil readings within the range of values encountered in commercial juices. The mixture was agitated vigorously to disperse d-limonene then diluted in a final step to 11.8 °Brix with sterile distilled water.

Inoculation and Storage of Juice. A 5 mL inoculum of hand-reamed fresh juices and peel washings was added to 195 mL of reconstituted juice. Containers were incubated at 4.5 ± 0.5°C. At intervals during storage serial dilutions were made in 0.1% sterile peptone broth and replicate plate counts were enumerated on orange serum agar (OSA) (Difco Laboratories, Detroit, MI) for total plate count and acidified potato dextrose agar (APDA)(Difco Laboratories, Detroit, MI) for yeast and mold count. All microbial experiments were replicated at least once.

Polymer Impact on Microbial Numbers. Juice with various d-limonene levels was prepared as described in the previous section. Sterile strips of each of the 4 polymers were added to separate flasks of juice. Polymer surface to juice volume ratios were twice the value found in standard gable-top citrus containers (1.89 L). The juice was incubated at 4.5 ± 0.5°C. OSA and APDA samples were taken periodically as described in the previous section.

Results and Discussion

Sensory Study. The higher surface-to-volume ratio between the polymer and juice was intended to approximate absorption losses expected from storage periods twice as great as those actually used. Consequently, experimental absorption at 3 weeks was intended to reflect retail absorption at 6 weeks.

Moderate oil juice in contact with LDPE for 3 weeks produced sensory scores significantly different from polymer free controls. D-limonene content was reduced by 43% in LDPE, 8% in nylon and less than 1% in PET and EVOH samples. Responses appeared consistent with absorption losses of d-limonene. However, sensory criticisms described the LDPE-treated juices as fermented, musty, yeasty and other descriptors consistent with spoilage. Comments suggesting a weakened flavor, as might be expected from absorption-biased flavor, were absent. The findings suggested that polymers other than LDPE did not influence flavor

A 2 week storage test was performed with LDPE-treated juices. Although d-limonene absorption was similar at two week (41%) and 3 weeks (43%), no sensory differences were observed between treated samples and controls. Remaining LDPE-

samples (high oil, low oil and thermally abused) were stored for 2 weeks. All other polymers/treatment combinations were run for 3 weeks. Sensory differences between the control and polymer-treated samples were not significant for any of the systems examined.absorption. Controls were free of polymer strips but were otherwise subjected to all treatment and storage steps described above.

These findings support olfactory (4) and sensory (12) studies in suggesting that intrinsic citrus flavor is not seriously affected by volatile absorption. However, the relationship between LDPE and microbially mediated flavor was not clear-cut. The possibility that microorganism survived the LDPE-sterilization step was explored. However, plate counts from all polymers were free of CFU's. The recurrent theme of high microbial numbers in LDPE-treated samples suggested some microbially restrictive agent may have been absorbed into the polymer. D-limonene was the likely candidate due to its predominance among citrus volatiles, its clear-cut removal through absorption and the known antimicrobial influence of related terpenes.

Microbial Studies, D-limonene and Microorganisms. The low pulp citrus concentrate used for juice in this study was essentially free of microorganisms. Therefore, juice samples were spiked with 5% hand-reamed orange juice which had been agitated in a container with the extracted peel fractions. It was hoped this effort would provide an inoculum of organisms likely to be encountered in commercially extracted juice.

D-limonene levels ranged from approximately 0.0001 - .0035%. The lower value represented residual peel-oil in the concentrate used for this study. The upper limit of this range represents the maximum Scott Oil value for grade A reconstituted frozen orange juice. Both APDA and OSA counts were significantly affected by d-limonene content by the 8th day in storage (Fig. 1). Microbial trends appears to largely reflect the impact of d-limonene on yeast and mold since OSA (total plate count) and APDA (yeast and mold) counts were statistically indistinguishable for all but the earliest dates in storage. The early demise of bacteria apparently cannot be blamed on d-limonene since bacteria was also absent in the control (0.001% d-limonene) within the first few days of storage. This early storage decline in certain microbial strains in refrigerated orange juice have been documented as a natural occurence by other researchers (15, 16).

These findings show d-limonene exerts a strong influence over microbial proliferation within the range of concentrations acceptable for grade A citrus juices. Considerable additional research is needed to understand the mechanism of inhibition and the spectrum of sensitivities found among various organisms. However, the pattern of inhibition shown in Fig. 1 and the diminished shelf life of LDPE-containing samples in sensory tests discussed previously, both suggest that volatile absorption can impact the shelf life of commercial pasteurized citrus juices.

Microbial Studies, Plastics and Microorganisms. D-limonene studies alone did not provide a vivid picture of the impact individual polymers made on microbial growth during the course of storage. In order to study the microbial response to individual polymers, juices were formulated with d-limonene values ranging from 0.001 to 0.0045%. The upper limit represents the maximum Scott Oil value for grade B

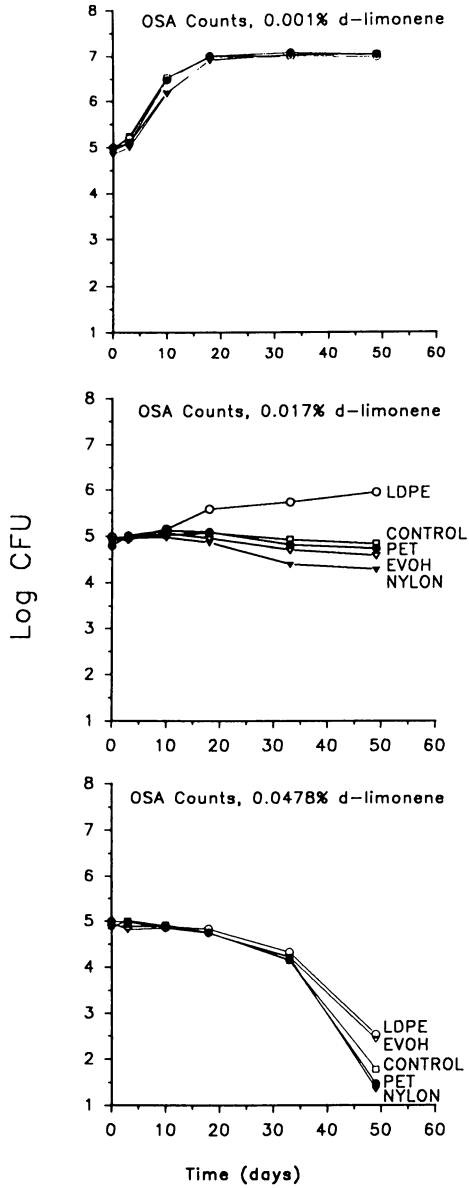

Fig. 1--The effect of d-limonene content on yeast and mold counts (ADPA) and total plate count (OSA agar) in orange juice stored at 4.5 ± 0.5°C.

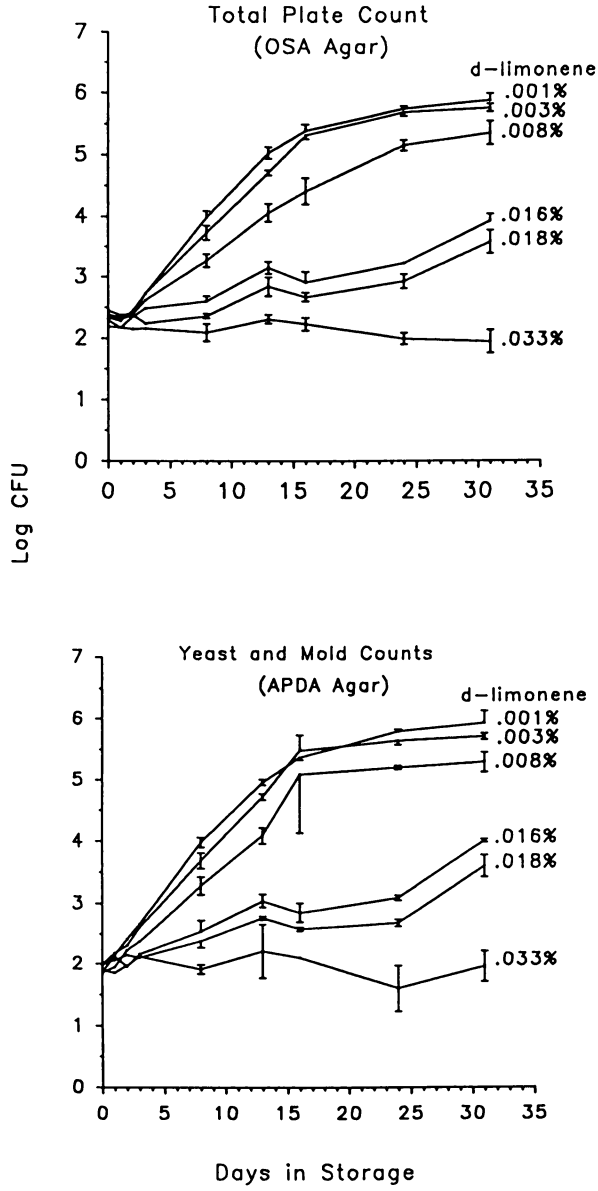

Fig. 2--The effect of packaging polymers on yeast and mold counts in orange juice stored at 4.5 ± 0.5°C containing various d-limonene levels.

reconstituted frozen orange juice. It is also a value selected by some fresh juice manufacturers. Fresh juice, unlike frozen reconstituted orange juice, does not currently consider grade based on Scott Oil values. As in flavor testing, sterile polymer strips were added to the juice at twice the polymer surface-to-volume ratio found in commercial gable-top containers. Microorganisms were monitored with time. Growth curves for low, medium and high oil juices are given in Fig. 2.

Microbial counts were statistically indistinguishable for all samples at the lowest d-limonene content. This indicates that LDPE did not harbor viable microorganisms or exhibit some previously unrecognized property which would account for the accelerated spoilage observed in sensory tests.

At the intermediate (0.017%) d-limonene content, LDPE-treated juices contained 10 to 100 times more organisms than juices containing other polymers or the polymer-free control by the end of study. Higher microbial numbers are consistent with the greater absorption tendency of d-limonene by LDPE. These results show that volatile absorption by packaging is extreme enough to influence microorganisms at peel oil levels typical of reconstituted frozen concentrated orange juice.

With rare exception, lowest microbial readings were found in juices containing nylon. Overall, microbial counts were significantly ($p \leq 0.05$) lower in nylon-containing juices than in polymer-free controls by the end of study. This phenomenon is difficult to reconcile with the simple premise that d-limonene level is the only polymer-dictated determinant of microbial number, since nylon absorbs more d-limonene than any material except LDPE. Nylon's role in microbial inhibition should be studied in greater detail in a separate study. Verification of its inhibitory role would indicate proper selection of packaging materials could confer greater shelf stability to orange juice through some currently unrecognized mechanism.

Conclusions

The impact of volatile absorption on orange juice flavor was limited to the acceleration of spoilage off-flavors by LDPE. There was no evidence that flavor absorption directly altered sensory characteristics of the juice through general or selective absorption of volatile compounds. Microbial growth was inhibited by d-limonene addition. Presumably this was through direct inhibition by d-limonene; however, the possibility that oxidized or hydrolyzed derivatives of d-limonene were responsible cannot presently be ruled out. Treatments containing nylon had significantly fewer microorganisms than controls. This finding should be explored through additional research.

Although this research suggests that barrier containers with non volatile-absorbing food contact surfaces will not result in improved flavor, they may add an additional measure of shelf life to pasteurized and fresh juices through supression of microorganisms..

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Chapter 19

Off-Flavor Development of Apples, Pears, Berries, and Plums Under Anaerobiosis and Partial Reversal in Air

Daryl G. Richardson[1] and Manit Kosittrakun[2]

[1]Department of Horticulture, Oregon State University, Corvallis, OR 97331–7304
[2]Department of Horticulture, Kasetsart University, Bangkok, Thailand

Responses of several fruit types to static N_2 or flowing streams (10 mL/ min) of low O_2 concentrations at 0°C or 20°C were investigated. Large differences in anaerobic responses were observed between varieties of the same fruit type. At 0°C 'Granny Smith' apple and 'd'Anjou' pear did not synthesize detectable off-flavors for at least 20 days in N_2 , whereas 'Golden Delicious' apple and 'Bartlett' pears developed off-flavors after only 5-8 days. At 20°C, both varieties of apples and pears synthesized off flavors in N_2, but 'Anjou' pear and 'Granny Smith' apple produced much less. Although off-flavor, ethanol and acetaldehyde increased under anaerobic conditions, the reversibility of off-flavor without much change in tissue ethanol or acetaldehyde, casts doubt on any direct connection between these compounds and off-flavor. 'Blue Jay' blueberries, 'Amity' red raspberries, 'Marion' blackberries, and 'Italian' plums were placed in static N_2 or continuous flow (10 ml/min) low O_2 (0.3 to 4.3%) at 0°C. For all fruits treated, accumulated CO_2 and headspace ethanol increased with time in static N_2. Blueberries, red raspberries, blackberries and plums could be kept in anaerobic conditions at 0°C for up to 9, 7,4, and 5 days, respectively without developing off-flavors. Storage beyond these periods for a few days resulted in slight off-flavors which dissipated after transferring fruits to air cold storage for 3 days. Longer anaerobic storage resulted in off-flavors which did not completely dissipate. In low-O_2 storage conditions, headspace ethanol increased with time in storage and with decreasing O_2 concentration. Small fruits held in 1.3% O_2 and plum kept in 0.5% O_2 for 10 days developed slight off-flavors, but recovered within 3 days upon transfer to air. While off-flavors could be at least partially reversed in air, changes in acetaldehyde or ethanol were very slight and these two compounds could not account for the changes in off-flavor.

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Controlled atmosphere (CA) storage has long been used successfully to extend postharvest life, and maintain quality of certain horticultural commodities, especially apples and pears. However, its potential application as an alternative to chemical fumigation to control insects of quarantine importance has not been thoroughly investigated. Storage in low-O_2 (1.0 to 1.5%) atmospheres with and without CO_2 (0 to 2.0%) resulted in better retention of flesh firmness and titratable acidity in apples (1,2,3,4,5,6) and better dessert quality and suppression of superficial scald and stem-end decay in pears (7,8,9). Use of CA as a quarantine treatment against insects in grains and stored products has been also well documented (10,11,12). Low oxygen concentration was more important in reducing the time to kill insects than CO_2 concentration (13). Low-O_2 ($\leq$1.5%) storage may offer a means to retain fruit quality and yet act as a biocide for insects on the fruit (14). Similarly, modified atmosphere packaging is being used or considered for several commodities and matching the film permeation rates to the rates of respiration of the commodities at the temperatures to which they may be exposed can create some problems. Exposure of fresh produce to high CO_2 or low O_2 beyond tolerance limits may increase anaerobic respiration and the consequent accumulation of acetaldehyde and ethanol, and may cause off-flavors (15). However, aeration following storage or packaging in such conditions may diminish the undesired flavor (16).

Due to extremely fragile structure and high rate of respiration, the postharvest life of most small fruits is relatively short. In addition, berry shelf life is limited by fungal rots. Storage of machine-harvested blackberries in 20% and 40% CO_2 at 20°C for up to 2 days maintained raw and processed quality (17). CO_2-enriched atmospheres could reduce postharvest decay development in blueberries during and after cold storage (18). Rapidly cooling fresh blueberries to a temperature just above freezing and maintaining that temperature appeared best for suppressing decay development and preserving the marketability of the fruits (19). Temperatures near 0°C were found to be the best for storing and handling blueberries and raspberries (20,21). Generally, small fruits are held at 0° to 5°C in 5 to 10% O_2 with 15 to 20% CO_2 during transport (22). Machine- and hand-harvested raspberries and blackberries kept in 5% CO_2 + 2% O_2 or 10% CO_2 + 2% O_2 at 0° or 5°C for 14 days had lower mold counts and better titratable acid and soluble solids retention, resulting in higher sensory panel rating when compared with those stored in air (23). Fruit quality of hand-harvested 'Climax' rabbiteye blueberries held in 20% CO_2 + 5% O_2 at 5°C for 42 days was as good as that of freshly harvested berries, whereas 'Woodward' blueberries stored under the same condition for 21 days exhibited a slightly fermented off-flavor (24). Interestingly, flavor of machine-harvested 'Climax' blueberries stored in N_2 did not change significantly, whereas that of 'Woodward' fruits became tart and fermented when berries were stored in N_2 for 21 days.

'Victoria' plums could be stored for 4 weeks or longer in 1% O_2 and less than 0.2% CO_2 at -0.5°C (25). Interrupting storage by 2 days at 18°C in air after day 16 resulted in further extension of storage life and reduction of internal browning and jellying. Exposure of 'Italian' plums to 20 to 60% CO_2 for 2 days at 7°C prior to storage at 0°C decreased softening and decay without off-flavors

being detected after 2 weeks storage despite accumulation of aldehyde and alcohols (26). Modified atmospheres created in sealed polyethylene bags, polyethylene box liners and in individually-wrapped fruits extended storage life of plums (27,28).

Studies of fruit responses to short-term anaerobic and low- O_2 storage conditions are important to modified atmosphere packaging. Thus, these experiments evaluated anaerobic and low-O_2 effects on physiology and flavor of certain small fruits and plums.

Materials and Methods

Apples *Malus domestica* cvs.'Golden Delicious' and 'Granny Smith'), pears (Pyrus communis cvs 'd'Anjou' and 'Bartlett'), 'Blue Jay' blueberries (Vaccinium spp.), 'Amity' red raspberries (Rubus spp.), 'Marion' blackberries (Rubus spp.) and 'Italian' plums (Prunus domestica) were harvested at commercial maturity and placed in static N_2 at 0° or 20°C or continuous flow (10 ml/min) low O_2 ranging from 0.3 to 4.3% in 1 pint glass jars at 0°C. Under anaerobic conditions, CO_2, O_2 and N_2, and headspace acetaldehyde and ethanol, and off-flavor development were determined daily. Anaerobic off-flavor development was determined by trained taste panel immediately after removing treated fruits from anaerobic conditions and again after 3 days in air. In low- O_2 storage conditions, treated fruits were analyzed similarly at 2-day intervals for 10 days. Off-flavor development of fruits in low O_2 was determined up to and including day 10 and after 3 additional days in air.

CO_2, O_2 and N_2 were measured with a Carle Model 311 gas chromatograph equipped with manually switchable Molecular Sieve 5A column (2 m x 3 mm O.D., 60/80 mesh) and a HayeSep R column (2 m x 3 mm O.D., 80/100 mesh) at 55°C and a thermal conductivity detector. Helium carrier gas flow was 30 ml/min. Acetaldehyde and ethanol concentrations were measured with the same gas chromatograph equipped with a Porapak Q column (2 m x 3 mm, 80/100 mesh) at 130°C and a flame ionization detector. Compressed air flow was 300 ml/min, hydrogen was 30 ml/min. Peak areas were quantified with a Shimadzu C-R3A digital integrator and calibrated with standard curves prepared from authentic acetaldehyde and ethanol. Off-flavor development was determined by a taste panel of 10 trained evaluators using an arbitrary hedonic scale of 0 to 8 with 0 = none, 2 = slight, 4 = moderate, 6 = strong, 8 = severe. A score of 1 was arbitrarily chosen as a level of off-flavors that might be regarded as undesirable by the taste panel.

Results

Pears and apples. The off-flavor development of Bartlett (Fig. 1) and Anjou pears (Fig. 2) and Granny Smith (Fig. 3)and Golden Delicious apples (Fig. 4) show the dramatic differences between varieties in response to static N_2 treatments at 0° and 20°C, and the reversibility of off-flavors upon return to air. Similarly, for Golden Delicious apple, headspace acetaldehyde and ethanol (Fig. 5), tissue acetaldehyde and ethanol (Fig. 6), and pyruvate decarboxylase and alcohol

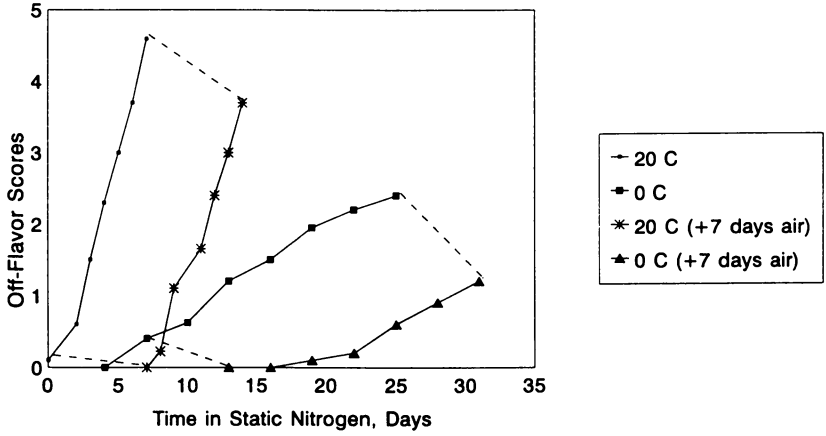

Figure 1. Off-flavor in Bartlett pears.

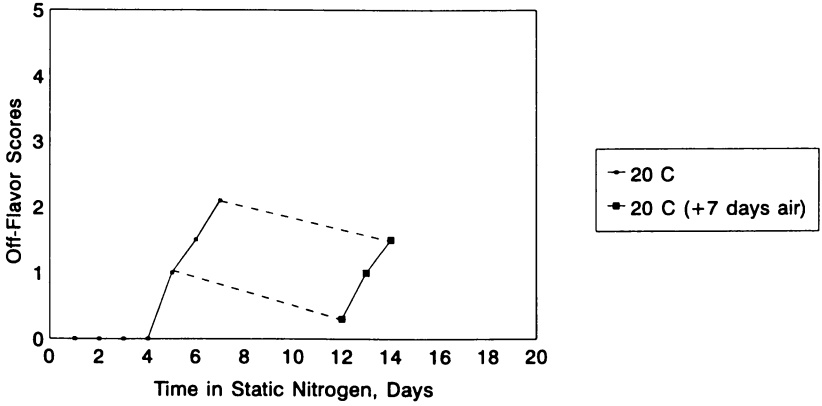

Figure 2. Off-flavors in Anjou pears (in static nitrogen, then air).

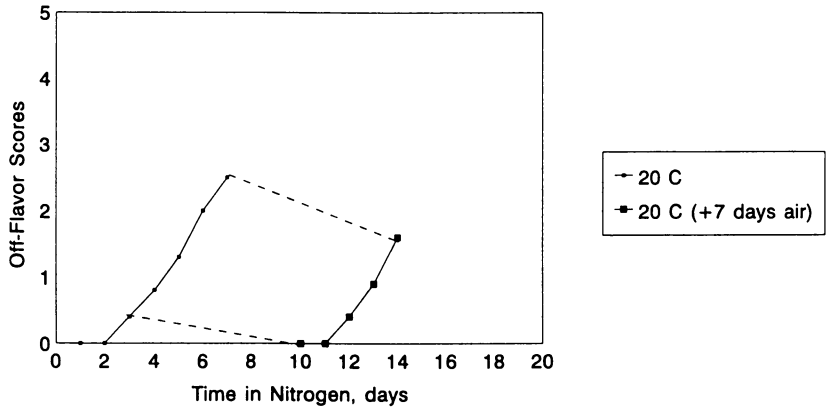

Figure 3. Off-flavors of Granny Smith apples (in static nitrogen, then air).

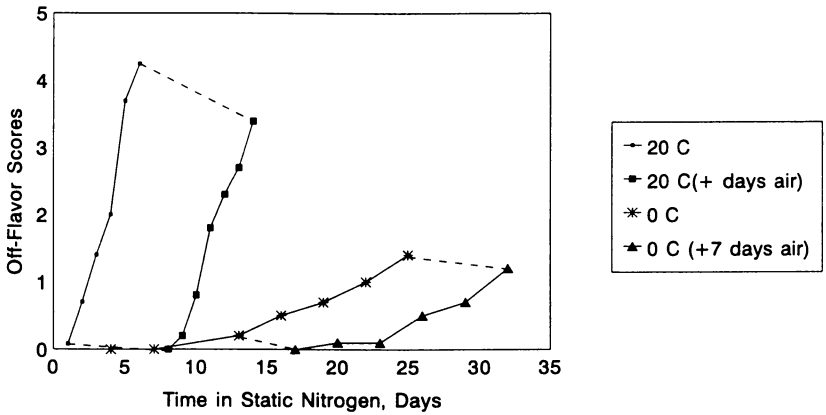

Figure 4. Off-flavors in Golden Delicious apples (in static nitrogen, then air).

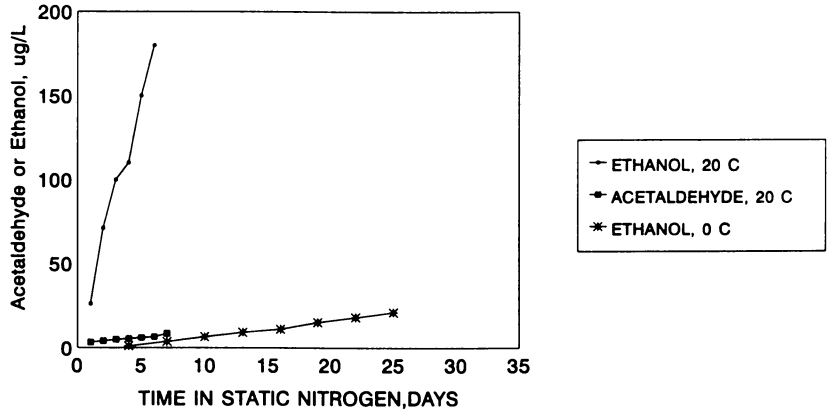

Figure 5. Headspace acetaldehyde and ethanol (Golden Delicious apples).

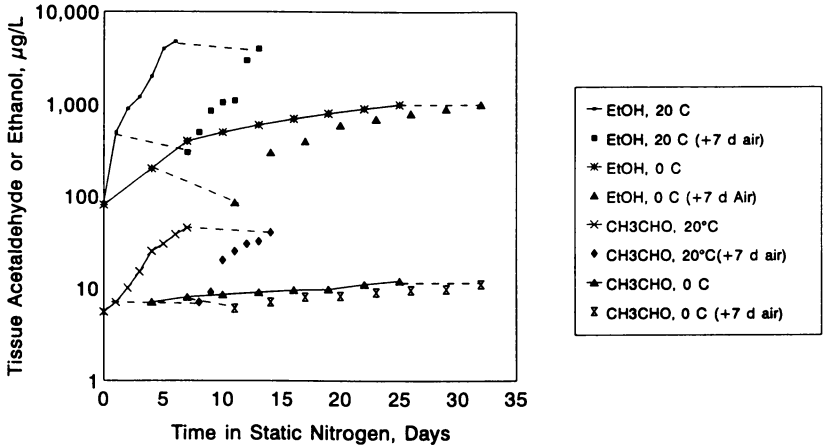

Figure 6. Tissue acetaldehyde and ethanol in Golden Delicious apples (in static nitrogen at 0 and 20 °C, then air).

dehydrogenase activity (Fig. 7) are shown as typical reactions to static N_2 at 0° and 20°C. There is not a good correlation between ethanol, acetaldehyde and off-flavor scores, especially as one includes the off-flavor reversibility upon return to air.

'Blue Jay' blueberries. Fig. 8. In static N_2 at 0°C, CO_2 accumulation increased steadily from 0.7% on day 1 to 17.3% on day 17 (data not shown), whereas accumulated headspace ethanol increased from 5 to 49 ug/l (data not shown). Headspace acetaldehyde could be detected on day 9 and slightly increased to 4 ug/l on day 17 (data not shown). The berries could be held anaerobically for 9 days without showing any off-flavors (Fig. 8). Ten to 14 days in static N_2 resulted in slight off-flavors with scores ranging from 1.3 to 3.3, but complete reversal was accomplished 3 days after transferring the berries to air. The berries held in N_2 longer than 16 days only slightly recovered from off-flavors when returned to air. Under low-O_2 environments, headspace ethanol increased with decreasing O_2 concentration (data not shown), but there were no differences in ethanol levels in 0.7 and 0.4% O_2. After 10 days in low-O_2 storage, slight off-flavors (scores 1.6 to 1.7) were detected in the berries in 0.7 and 0.4% O_2. Berry flavor became normal after subsequent holding in air for 3 days.

'Amity' red raspberries. Fig. 9. CO_2 accumulation in static N_2 at 0°C increased from 3.4% on day 1 to 9.2% on day 10 in parallel to an increase of accumulated headspace ethanol from 11 to 61 ug/l. The berries did not exhibit off-flavors when held in anaerobic conditions for up to 7 days, but started to develop off-flavors on day 8. However, the berries completely recovered from the undesired flavor after transfer to air for 3 days. Partial reversal (30 to 79%) was accomplished when the berries were in static N_2 longer than 8 days, and the off-flavor scores ranged from 0.3 to 2.3 following 3 days in air. In low-O_2 storage conditions at 0°C, headspace ethanol increased in proportion to decreased O_2 from 4.3 to 0.4%. After 10 days in storage, the berries held in $\leq$1.3% O_2 had slight off- flavors with scores ranging from 1.3 to 2.3. However, off-flavors disappeared after transferring the berries to air (data not shown).

'Marion' blackberries. Fig. 10. CO_2 accumulation increased with time in static N_2 at 0°C from 4.4% on day 1 to 18.4% on day 9, and accumulated headspace ethanol increased in the same fashion from 9 to 52 ug/l. Flavor of the berries held in anaerobic conditions for up to 4 days did not change when compared with the control. The berries showed slight off-flavors (scores 1.3 to 3.0) when kept in such conditions for 5 to 8 days, but complete reversal of off-flavors was obtained 3 days after the berries were transferred to air. Headspace ethanol could not be detected in 4.3% O_2 atmosphere. At lower O_2 levels, ethanol concentration increased with time in storage and decreasing O_2 concentration. However, ethanol levels in 0.7 and 0.4% O_2 appeared to be the same. Ten days in low-O_2 storage resulted in slight off-flavors (scores 0.6 to 1.9) in the berries kept in 1.3 to 0.4% O_2. Flavor of these berries became normal after transfer to air for 3 days.

'Italian' plums. Fig. 11. In static N_2 at 0°C, CO_2 accumulation increased steadily with time in storage from 1.4% on day 1 to 9.2% on day 10, and accumulated headspace ethanol increased from 6 to 47 ug/l. Fruits did not show off-flavors when kept anaerobically for up to 5 days, but had slight off-flavors

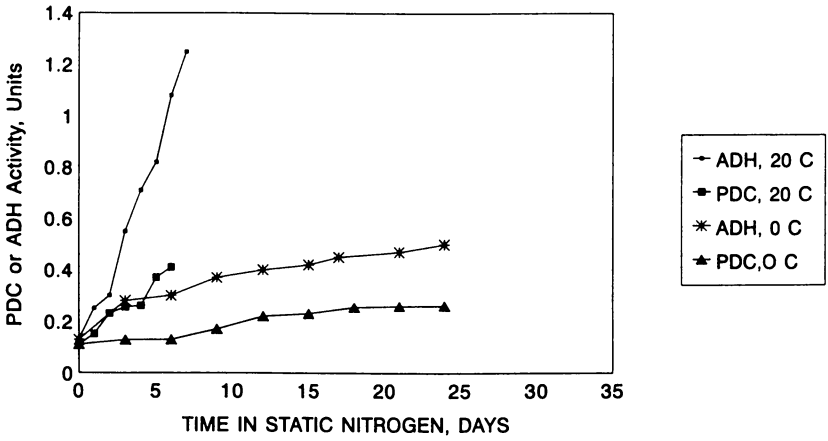

Figure 7. Golden Delicious apple pyruvate decarboxylase and alcohol dehydrogenase under anaerobiosis.

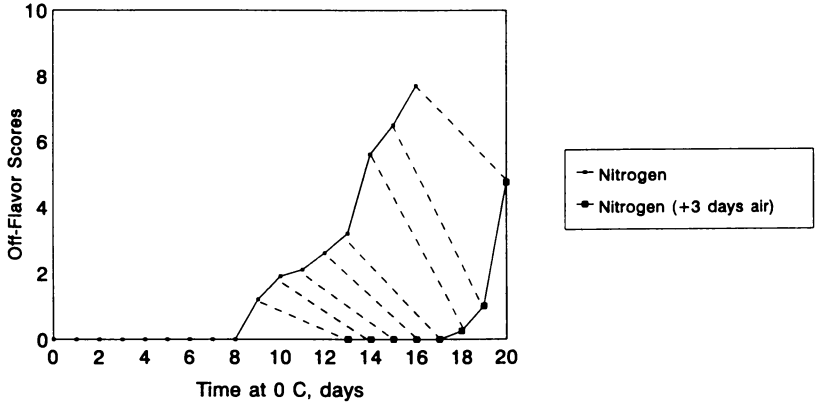

Figure 8. Off-flavor in Blue Jay blueberries (in static nitrogen, then air).

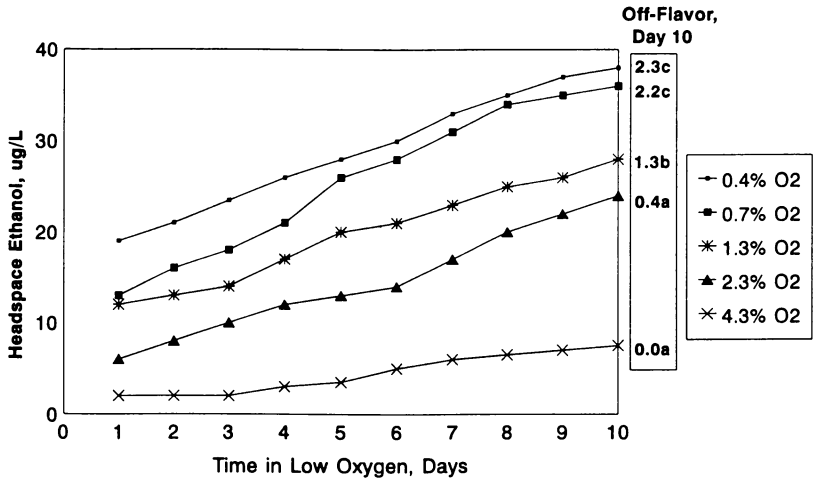

Figure 9. Headspace ethanol and off-flavor of Amity Red raspberry [low O_2 (10 ml/min.), 0 ° C].

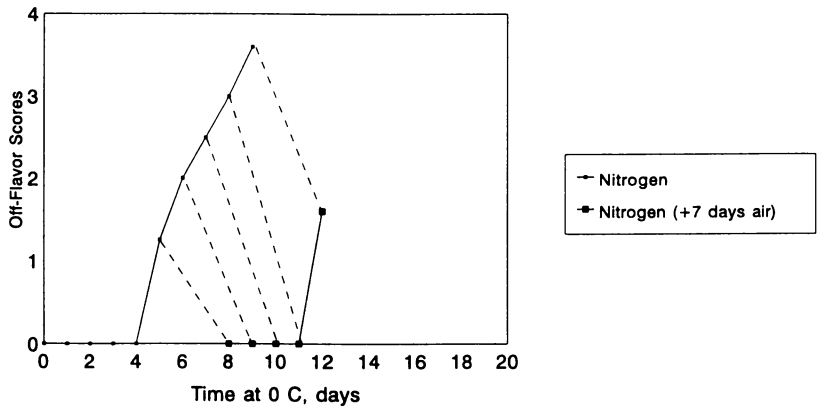

Figure 10. Off-flavor of Marion blackberries (in static nitrogen, then air).

(scores 1.3 to 2.7) when held in N_2 for 6 to 9 days. Complete reversal of off-flavors could be accomplished by placing the plums 3 days in air after fruits were kept anaerobically for up to 9 days. In flow-through low-O_2 storage (Fig.12), headspace ethanol was not detected in 3.4% and 1.7% O_2 atmospheres. Plums kept in O_2 at 1.1 to 0.3% increased ethanol levels with time in storage and decreasing O_2 concentration. After 10 days in storage, plums kept in $\leq$0.5% O_2 exhibited slight off-flavors (scores 1.3 to 2.3) which disappeared when fruits were subsequently held in air for 3 days.

Discussion

In anaerobic or partially anaerobic conditions, both CO_2 accumulation and headspace ethanol increased with time in storage for all fruits studied. This indicates that there was little, if any, inhibition by CO_2 on ethanol accumulation. CO_2 in the absence of O_2 was reported to inhibit anaerobic ethanol production in apples (29). In contrast, CO_2 enhanced ethanol accumulation in sweet potato roots (30). In addition, high CO_2 enhanced acetaldehyde production in persimmon fruit (31), but did not affect ethanol production in grapes (32). In all fruits but blueberries in our studies, accumulated headspace acetaldehyde was not detected {detection limit was 0.5 ug/l} at these storage temperatures of 0°C. Anaerobic ethanol accumulation in apples, pears, berries, and plums increased with time of exposure in static N_2 and this agrees with reports for blueberries and grapes (33,32).

Acetaldehyde and ethanol increased during apple and pear fruit ripening in air (34,35). However, both substances were present in only trace amounts under aerobic conditions, but increased markedly in anaerobic conditions (36,37) and our data agrees with this. Headspace ethanol in all fruits investigated increased with decreasing O_2 concentration. However, headspace ethanol was not detected in plums held in O_2 greater than 1.7%. Oxygen tolerance limits previously recommended for blueberries, raspberries and blackberries stored at 0° to 5°C were 5 to 10% O_2, whereas those for plums were 1 to 2% O_2. Our study suggests that the low oxygen tolerance should be about 1.5% O_2 for most berries, whereas for plums 1 - 2% O_2 which agrees with (38).

Exposure of fresh fruits and vegetables to O_2 levels beyond tolerance limits may increase anaerobic respiration and the consequent acetaldehyde and ethanol accumulation, causing off-flavors (15,16). 'Redhaven' peaches exposed to N_2 at 15.5°C for 6 days did not developed off-flavors (39). 'Wickson' plums stored in 1% O_2 at 15°C for 21 days exhibited off-flavors (40). There were varietal differences in response to anaerobic conditions (24) and our data clearly demonstrates this. `Woodward' blueberries developed off-flavors, but 'Climax' blueberries did not, when held in N_2 at 5°C for 21 and 42 days. 'Italian' plums could be stored anaerobically for up to 5 days without off-flavors being detected. Among small fruits used in this experiment, blueberries were the most tolerant to anoxia, followed by raspberries and blackberries. They could be kept anaerobically at 0°C for 9, 7 and 4 days, respectively without showing off-flavors. Holding fruits in N_2 beyond these periods for a few more days resulted in slight off-flavors which dissipated after 3 days in air. Fruit ethanol which accumulated during anaerobiosis

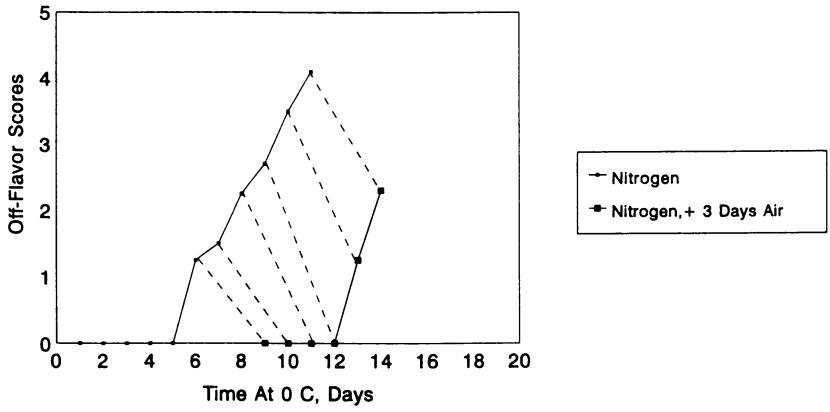

Figure 11. Off-flavor of Italian plums (under static nitrogen, then air).

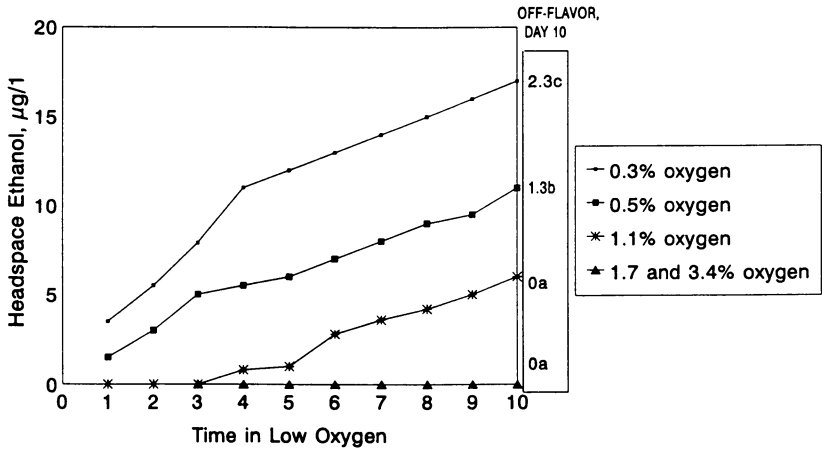

Figure 12. Headspace ethanol and off-flavor of Italian plums stored at 0 °C for 10 days in low oxygen.

or low-O_2 storage decreased only slightly when fruits were transferred to air possibly due to better diffusion and metabolic conversion (41,29). In these experiments, no visible physiological disorders were observed in fruits as influenced by anaerobic or low-O_2 atmospheres at 0°C . The reversibility of off-flavors evident in our study, while showing a general phenomenon, may not apply to all fruits. We have preliminary evidence showing that some off-flavors in fruits such as banana are, indeed, alcoholic in description, and thus would be expected to be closely linked to tissue levels of ethanol. The most likely mode of reversibility of off-flavor probably involves conversion of the offending compounds to esters or other modifications via oxidations upon return of the fruits to air. It is much less likely that these off-flavors simply diffuse away, because they should be more efficiently removed in the flow-through systems.

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Chapter 20

Vegetative Flavor and Methoxypyrazines in Cabernet Sauvignon

Ann C. Noble[1], Deborah L. Elliott-Fisk[2], and Malcolm S. Allen[3]

[1]Department of Viticulture and Enology, University of California, Davis, CA 95616
[2]Department of Land, Air, and Water Resources, University of California, Davis, CA 95616
[3]Ron Potter Centre for Grape and Wine Research, Charles Sturt University, Wagga Wagga, New South Wales 2650, Australia

Cabernet Sauvignon wines are characterized by berry and vegetative aromas. Winemakers have known for decades that young vines in cool climates and vigorous vines with dense canopies tend to yield very intensely vegetative wines. Only with the advent of a GC-MS method using isotopically labeled methoxypyrazines was it possible to conclusively identify and quantify the potent 2-methoxy-3-isobutyl pyrazine (MIBP) which had long been suspected to contribute to this bell-pepper-like aroma in wines. A review of sensory and chemical studies of Cabernet Sauvignon wine flavor is presented, including a recent study which in which the effect of vine vigor and light on wine flavor and methoxypyrazine levels was investigated. A strong correlation was found between high vine vigor, low light intensity in the canopy and the intensity of the vegetative aroma and flavor by mouth, with the concentration of MIBP.

In 1969, Buttery *et al.* identified 2-methoxy-3-isobutylpyrazine (MIBP) as the potent compound responsible for the distinctive odor of bell peppers (1). Since then, the "herbaceous" or "vegetative" aroma of Sauvignon blanc and Cabernet Sauvignon wines has been speculatively attributed to the presence of the "bell pepper pyrazine" (2). Intense bell pepper aroma is generally not desirable in these wines, thus there has been considerable interest in identifying the compound or compounds responsible for the vegetative aroma. Despite the number of investigators working on this topic, only in 1987 was the presence of MIBP conclusively identified in Sauvignon blanc wines and in 1989 in Cabernet Sauvignon. In this paper, steps in the partial solution of the "bell pepper aroma" puzzle will be presented.

Development of an Analytical Method

Using a simultaneous distillation-extraction technique, MIBP was identified in bell peppers by mass, infrared and UV absorption spectra by Buttery in 1969 (1), who noted that the important compound was "initially difficult to interpret" since the molecular ion was only 3.5% of the most intense ion. For these experiments, five kg

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of peppers were distilled to provide 10 mg of oil, of which MIBP constituted 6 to 16% of total peak area. The odor threshold of this potent compound in water is two parts per trillion (2 ng/l) (1, 3), thus Buttery and co-workers identified MIBP at concentrations approximately several thousand times above minimally detectable sensory levels. To identify MIBP at trace levels, which elicit intense bell pepper aromas, requires exhaustive concentration and sophisticated sensitive analytical methods. It is not surprising therefore that positive identification of MIBP at the trace concentrations at which it occurs in grapes and wine has taken two decades of flavor research.

Using pentane extraction, Bayonove *et al.* (2) very tentatively identified the presence of MIBP in Cabernet Sauvignon grapes. Analysis of 500 ml of Cabernet Sauvignon wine headspace revealed a distinct bell pepper aroma when the split GC eluate was sniffed, but no peak corresponding to a retention time of MIBP was found even in the wines with the most intense vegetative aromas (4). Similarly, despite exhaustive methylene chloride extraction of Cabernet Sauvignon wines which had distinctive vegetative aromas, no MIBP was found (5). Using Freon extraction and headspace techniques, MIBP, 2-methoxy 3-ethyl and 2-methoxy 3-isopropyl (MIPP) pyrazines were very tentatively identified in Sauvignon blanc grapes, since the concentrations were near the levels of sensitivity of the method (6).

Assuming that Slingsby's failure to find MIBP in vegetative Cabernet Sauvignon wines was due to poor extraction recovery, Heymann *et al.* (7) steam distilled wine to separate the volatiles from non-volatile phenols which co-eluted with MIBP. To increase recovery of the pyrazines, the pH of the wine was raised before distillation, and the distillate acidified, before concentration on a C_{18} cartridge for HPLC. However, this method which had a minimum detection level of 1.2 μg/l, is only useful for analysis of white wines with no skin contact; red wines and white wines aged in oak contain volatile phenols which co-elute with MIBP using either HPLC or conventional gas chromatography-mass spectrometry(8). A very important result of Heymann's research however, was the observation for the first time that both 2-methoxy-3-isobutyl pyrazine and 2-methoxy-3-isopropylpyrazine were very sensitive to light. After 120 hr exposure to fluorescent light, aqueous solutions of either MIBP and MIPP showed a loss of about 30% by photodegradation, whereas no changes occurred in controls held in the dark (7).

Successful identification of MIBP was finally achieved in 1987 with modifications to the analytical procedures which both increased sensitivity and eliminated interference of volatile phenolic compounds (9). Deuterium labeled MIBP was added as an internal standard to wine prior to steam distillation. The pyrazines were recovered from the distillate on an acidic ion-exchange resin which was extracted with methylene chloride for separation by GC. Subsequent identification and quantification were performed with selective ion monitoring mass spectrometry. The ensitivity of the method (0.15 ng/l or 0.15 ppt) was further enhanced by use of positive ion chemical ionization (CI) with ammonia as the CI reagent gas which ionizes only molecules with a significant basicity. With this elegant technique, MIBP was conclusively identified and quantified at a level of 35 ng/l in a New Zealand Sauvignon blanc wine (9). In addition, 6 ng/l of MIPP was also detected. In analyses of 22 Sauvignon blanc wines, MIBP was the major methoxypyrazine, with MIPP found in half of the wines at lower levels. The ratio of MIBP to MIPP was fairly constant at 7:1. In three wines, 2-methoxy-3-*sec* butylpyrazine (MsecBP) was found at levels below 1 ng/l (10). Analysis of Cabernet Sauvignon wines found higher levels of MIBP and MIPP than those observed in Sauvignon blanc, with only trace amounts of MsecBP (11).

Factors influencing Intensity of Vegetative Aroma

Vine Vigor and Viticultural Practices. For many years, vineyard and winery personnel had reported anecdotally that vineyards which had very vigorous vines, with extensive vegetative growth, yielded wines with intense vegetative or bell pepper aromas. These sensory observations were subsequently analytically documented in both Cabernet Sauvignon and Sauvignon blanc varieties. Cabernet Sauvignon vines were cultivated to provide shaded fruit, shaded leaves, shaded leaves and fruit, and the unshaded control (12). Grapes and wines produced from all shaded treatments were found to be more intense in "vegetative" aroma than those from the open, unshaded canopies which had higher light exposure. In a similar experiment with Sauvignon blanc vines, increasing light exposure in the fruiting zone by leaf removal reduced the intensity of the "vegetative" wine aromas (13).

Consistent results were found in Cabernet Sauvignon wines made from vineyards with different soils. Using Partial Least Squares analysis (PLS), data characterizing the soil at each vineyard site was related to the sensory descriptive analyses of the wines, demonstrating an association between wines with higher intensity of vegetative aroma and flavor by mouth and soils with higher water holding capacity (14). Conversely, fruitier wines, high in berry aroma and flavor were associated with older, gravely soils with poor water holding capacity. Two wines which were produced at the same winery from vines grown in very different soil types yielded extremely different flavors; one (#1) was characterized by a fruity (berry) aroma and flavor by mouth while the second (#2) was high in bell pepper, soy and vegetative aromas and in vegetative flavor. Consistent with the PLS model, soil at site #1 was shallow, sandy, and nutrient-poor with a low water holding capacity, and wine #1 was quite fruity. In contrast, the vegetative wine #2 was produced from a deep, clay-rich soil, high in nutrients and water holding capacity (14). Nutrient poor soils with low water holding capacity yield less vigorous vines which have very open canopies, exposing the fruit to high levels of light. Visual inspection of the two sites confirmed that vines at site #1 were low in vigor, providing extensive light exposure to the fruit, whereas at site #2 vines were virtually a jungle with dense canopies limiting berry light exposure (Elliott-Fisk unpublished).

Unfortunately, in these experiments, no determination of MIBP was made nor was available light in the canopy measured in the fruiting zone. Despite that, given that MIBP photodegrades even at low light intensity (7), it was speculated that the reduction in the vegetative aroma in the studies described above could be a result of lower MIBP levels in light exposed fruit (15).

Effect of Climate, Vine Age and Maturation. Other factors influencing the intensity of vegetative aromas in Cabernet Sauvignon wines have been informally reported by both Bordeaux and California wine makers. Cooler climates, younger vines and less ripe grapes were both reputed to yield Cabernet Sauvignon wines which were high in bell pepper or vegetative notes. Randomly sampling berries in a vineyard readily shows that green or less ripe berries are higher in the bell pepper note than riper (higher sugar content) grapes on the same vines.

Descriptive sensory analysis of 21 commercial Cabernet Sauvignon wines quantitatively corroborated some of these claims (16). The intensity of the "green bean" aroma and vegetative flavor by mouth was highly significantly negatively correlated with the age of the grape vines. Negative correlations were also found between the average temperature of the region and intensity of eucalyptus aroma and vegetative flavor by mouth. However, no MIBP determinations were made on these

wines, since the analytical procedure for analysis of red wines had not yet been developed.

Factors Influencing Methoxypyrazine Concentration

Effect of Climate and Maturation. Consistent with the informal reports and Heymann's quantitative sensory analysis, the level of MIBP in Cabernet Sauvignon and Sauvignon blanc berries was shown to be highest in grapes from cooler climates (10, 11). Futher, MIPB was found to generally be higher in Cabernet Sauvignon than Sauvignon blanc wines. MIBP was 10 to 20 times higher in Australian Sauvignon blanc fruit from cool areas than in grapes from a warmer region. Additionally, MIBP was highest at véraison, the point at which grape sugar and pigment synthesis begins. For Sauvignon blanc, the final grape concentration at harvest was only 2% of that at véraison (30 ng/l). Expressing the Sauvignon blanc results on a per berry basis to eliminate the effect of dilution as the berries expand on ripening showed a 96% reduction in MIBP at harvest (10) For Cabernet Sauvignon, MIBP decreased from 75 ng /l at véraison to 5 ng /l at harvest (11).

Effect of Light and Vine Vigor. The definitive study establishing the relation between light within the canopy, MIBP concentration and wine aroma was recently performed in a Sonoma Valley Cabernet Sauvignon vineyard owned by Simi Winery. Standard soil analyses and soil horizon definitions were defined in soil pits at each of five sites selected for maximum soil diversity. Vine vigor was estimated by winter pruning weights. The fraction of light available in the fruiting zone was measured with two light sensors prior to harvest. Light measured as photosynthetically active photon flux density (PPFD) by a quantum sensor within the canopy was reported as the fraction of total available light. Descriptive analysis of the wines was performed by standard procedures to profile the wine flavors. The concentration of methoxypyrazines were determined using deuterium labeled MIBP and MIPP standards and selective ion monitoring with positive ion chemical ionization mass spectrometry similar to the distillation procedure described by Lacey *et al* (10).

Differences in soil water holding capacity, drainage and nutritional content influenced vine vigor, which in turn affected light exposure in the fruiting zone and subsequent wine flavor and methoxypyrazine concentrations, as shown in Figure 1. MIBP was found at suprathreshold levels in all wines, ranging from 2.8 to 37 ng/l. MsecBP was found at levels below its sensory threshold, while there was no detectable MIPP. Site 12 UP had the highest overall water holding capacity (WHC), while 25B LNE had the lowest. 12 UP was a very old, leached soil, with very low pH (3.9) and consequently unbalanced mineral status, but the high WHC resulted in vigorous vines. 12 LO was a shallow, well drained, bouldery, soil, low in nutrients,which yielded vines with lower vigor (Figure 1). 25B LNE soil was a sandy clay loam with good drainage, yielding less vigorous vines, which were comparable to those at site 12 LO. 25B LSW (a bouldery soil) and 25B UP (high in pebbles) yielded vines which were intermediate in vine vigor (Elliott-Fisk, unpublished).

Site 12 UP, which was highest in vine vigor, had the lowest available light in the fruiting zone, and correspondingly yielded wines dramatically higher in MIBP (37 ng/l) and in intensity of vegetative aroma (Figure 1). Sites 25B UP and 25B LNE which had the highest light exposure had the lowest vegetative aroma and lowest levels of MIBP (6.5 and 2.8 ng/l, respectively). The flavor profiles of the three most different wines are shown in Figure 2. The intense vegetative aroma and flavor by mouth of the wines from 12 UP contrast with the low levels of these attributes in 12

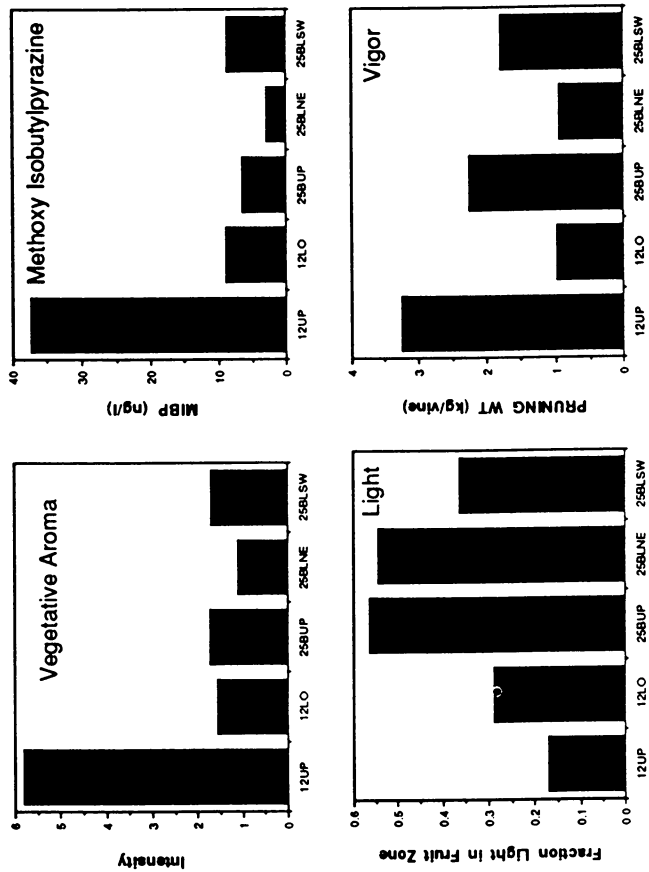

Figure 1. Wine and vine data for five Cabernet Sauvignon sites. Vegetative Aroma Intensity (n = 15 judges x 2 reps) and concentration of 2-methoxy-3-isobutylpyrazine (ng/l) for wines. Fraction of photosynthetically active available light in the fruiting zone and vine vigor estimated by pruning weight (kg/vine).

LO and 25B LNE, which are higher in berry aroma and fruity (berry) by mouth, as well as the rose and chocolate aromas.

Partial least squares analysis was performed to model the flavor ratings (Y-matrix) using soil variables, vine vigor and available light, and grape and wine composition, including the methoxypyrazine concentrations (X-matrix). In Figure 3, are shown the factor loadings for the sensory terms and for variables including viticultural, soil and berry/wine composition. Site 12 LO is located in the fourth quadrant, where high vegetative aroma and flavor by mouth are associated with high levels of MIBP and MsecBP, and high vine vigor. 12 LO is separated from the other wines on the first factor, based on its lower light level and fruity character, as well as higher vine vigor and vegetative notes.

Clearly these data suggest that light, and possibly vine vigor, directly or indirectly, affect the level of MIBP which in turn affects the intensity of the bell pepper aroma in wine. These data do not establish that MIBP is the sole compound responsible for the vegetative aroma in these wines, however the correlation between MB and vegetative aroma intensity was significant ($r = 0.90$, $p<0.05$). Similarly, highly significant correlations between MIBP concentration and vegetative "varietal" aroma intensity have been found for commercial Sauvignon blanc wines and between vegetative aroma intensity and concentration of MIBP spiked in a neutral wine (17).

Vegetative aroma notes other than bell pepper are also reported in both Cabernet Sauvignon and Sauvignon blanc. Only recently has a compound which produces a vegetative aroma described as "boxwood" or "cassis bud" (or cat urine) been identified in Sauvignon blanc wine. Concentrations of 4-mercapto-4-methyl-pentan-2-one up to 10 ng/l were found in wine, but none was found in unfermented must (18, 19) The structure of the precursor, as well as the chemical or enzymatic mechanisms for its formation are unknown. Dimethyl sulfide, which has a distinct asparagus aroma, has been found at relatively high levels in both Sauvignon blanc and Cabernet Sauvignon wines (20).

What controls synthesis of MIBP?

Alkyl pyrazines occur widely in roasted or heated systems, but alkoxypyrazines are not produced by maillard browning reactions or caramelization. Biosynthesis of alkoxypyrazines occurs in a variety of plant systems, such as peas, potatoes, beets and in molds and bacteria, but seldom in mammals (21). In bell peppers and jalapeno peppers, and in peas MIBP contributes to the distinctive vegetative aroma. Molds and bacteria synthesize MIPP which has been identified as the source of a moldy or potato-like spoilage odor (22), while 2-methoxy-3-ethylpyrazine is associated with a raw potato aroma (23).

Murray *et al.* (24) proposed a pathway for the synthesis of alkoxypyrazines which required amidation of an amino acid followed by condensation with glyoxal, then methylation. Although the sequence is reasonable chemically, enzymes required for amidation have not been found. With radioactive labeling, Cheng *et al.* (25) demonstrated that MIPP is synthesized from endogenous valine, glycine and methionine by *Pseudomonas perolens.* However, the site and pathway for synthesis of MIBP in grapes are unknown.

Preliminary experiments suggest that MIBP, MIPP and MsecBP are not localized in the skins alone: clarified juice, prior to fermentation contained about 50% of the MIBP found in wine after fermentation on the skins, suggesting MIBP is located both in the pulp and the epidermal tissue (11). Highest levels of MIBP are found at véraison (10). As the grape ripens and swells during maturation, the concentration per berry decreases. Whether this decrease is due to photodegradation and/or cessation of

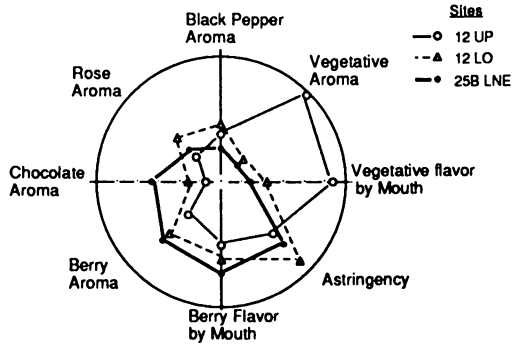

Figure 2. Mean intensity ratings for three Caberent Sauvignon wines. The center of the figure represents low intensity with the distance from the center to the intensity rating corresponding to the relative intensity of each wine for the attribute (n = 15 judges x 2 reps).

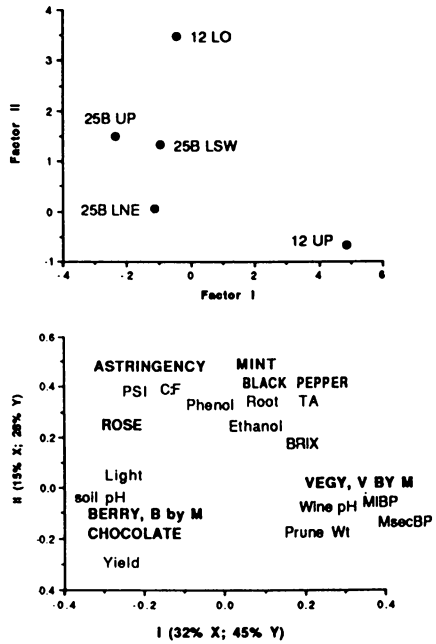

Figure 3. PLS of Cabernet Sauvigon sensory and non-sensory data. Factor scores (top) and variable loadings (bottom) for the soil, viticultural, berry and wine composition (X matrix) and sensory ratings (Y matrix, in bold) for Factors I and II.

synthesis of MIBP cannot be determined from any of the present information. Certainly when extreme differences in light exposure occur, photodegradation must contribute to the decrease in MIBP. However, with experimental vines followed over the growing season, the canopy should not change appreciably from véraison to harvest, suggesting that light in the fruiting zone is not relevant or at least is less imporant in reducing MIBP levels. The site of methoxypyrazine synthesis is not known. Possibly, if synthesis occurs in leaves with subsequent transport to berries, decrease in MIBP could result from both photodegradation and volatilization, however Cabernet Sauvignon vines don't smell like bell peppers!

Conditions favoring vine vigor, such as available water and nutrients in the soil, result in root tip proliferation and synthesis of cytokinins, which in turn stimulate the production of auxins in shoot tips. Hormonal control of the production of MIBP could favor its synthesis in the shoot growth phase of vine growth, and turn it off when the plant shifts from growth phase to the reproductive phase.

Conclusion

The distinctive bell pepper aroma of Cabernet Sauvignon wines is elicited by 2-methoxy-3-isobutylpyrazine (MIBP). Concentrations of MIBP are highest at the onset of ripening and decrease as the fruit matures. Levels of this light-sensitive compound are lower in wines from less vigorous vines with open canopies than from dense vigorous vines which restrict light exposure in the fruiting zone. The relative importance of temperature, light and fruit maturity in determining the concentration of methoxypyrazines is unknown. To determine factors which regulate synthesis of MIBP, the pathway needs to be defined in plants.

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Chapter 21

Dynamic Headspace Gas Chromatography–Mass Spectrometry Analysis of Volatile Flavor Compounds from Wild Diploid Blueberry Species

David W. Baloga[1], Nicholi Vorsa[2], and Louise Lawter[1]

[1]Sanofi Bio-Industries, Inc., Flavor and Fruit Division, 2607 Interplex Drive, KOR-Center B, Neshaminy Interplex, Trevose, PA 19053
[2]Blueberry and Cranberry Research Center, Department of Plant Science, Cook College, Rutgers University, Chatsworth, NJ 08019

Variation in flavor components of seven putative diploid progenitor species *(Vaccinium boreale, V. corymbosum, V. darrowi, V. elliottii, V. myrtilloides, V. pallidum, and V. tenellum)* of the North American domesticated blueberry (highbush, rabbiteye and lowbush) were examined. Samples from one to four populations per species were analyzed. Volatile flavor compounds in the juice headspace of blueberries from each plant were analyzed by dynamic headspace gas chromatography-mass spectrometry DH-GC-MS. More than 30 volatile compounds were identified and quantified. Analysis of variance was employed to determine significance of species effects. Multivariate analysis was applied to flavor profiles, titratable acidity, soluble solids. The seven wild blueberry species were differentiated by principal component analysis into six groupings which correlate with taxonomic groupings based on allozyme data.

Species of the genus *Vaccinium* L. sect. *Cyanococcus* represent the "true clustered-fruited" blueberry, and are found from subtropical to temperate regions of North America. Three species of blueberry are cultivated commercially. Two species are tetraploid, Highbush and Lowbush, and one is hexaploid, Rabbiteye. The majority of the North American blueberry crop is used for industrial purposes and is individually quick frozen (IQF) for storage. Blueberries are particularly popular in North America and Scandinavia as a breakfast or dessert food i.e fresh fruit, cereal topping, bagel, muffin, pancake, waffle, pastry filling, yogurt and ice cream topping or in syrups. In the United States blueberries are commercially grown in the eastern Atlantic (Nova Scotia, New Brunswick, Maine, New Jersey, North Carolina, Arkansas, Alabama, Georgia and Florida), in the midwest (Michigan, Minnesota), and in the Pacific northwest (Oregon, Washington and British Colombia). Mechanical and manual harvesting of the mature fruit is typically conducted in the months of July and August. Availability of fresh

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blueberries is limited to the May through August harvest season. Fresh blueberries are not typically sold as a varietal fruit, however, some food manufacturers have recently included the descriptor "wild" on blueberry-flavored product labels with the inference of more flavor intensity.

Seven wild diploid blueberry species have been taxonomically defined as those that have given rise to all the blueberry polyploid species native to North America (1). These seven diploid progenitor species are *V. boreale* Hall & Alders (Vb), *V. corymbosum* L. (Vc), *V. darrowi* Camp (Vd), *V. elliottii* Chapm. (Ve), *V. myrtilloides* Michx. (Vm), *V. pallidum* Ait. (Vp), *V. tenellum* Ait.(Vt).

The volatile flavor compounds of the highbush blueberry (*V. corymbosum*) (2,3,4), bog blueberry (*V. uliginosum* L.) (4,5), rabbiteye blueberry (*V. ashei* Reade. cv. Tiftblue) (6), and the "European blueberry", bilberry or whortle berry, (*V. myrtillus* L.) (7,8) have been previously described. Determination of the flavor components of the blueberry juice in these studies involved isolation of volatiles by either direct solvent extraction, continuous liquid-liquid extraction, simultaneous distillation-extraction or steam/vacuum distillation. Although these isolation methods are useful in comprehensive identification of fruit flavor constituents, they require a relatively large sample size and involve time consuming procedures. In addition, the comparison of flavor volatiles (isolated and quantified by different methodologies) among species may not be justified (9).

Organoleptic differences among blueberry species grown under similar climatic and environmental conditions were noted from field observations at Rutgers Blueberry/Cranberry Research Center (Chatsworth, NJ). The objective of this study was to examine in some detail the differences in flavor profile of the seven progenitor wild diploid North American blueberry species and to determine if any taxonomic correlation could be made. Currently, berry sugar and acid levels, size, color and texture are primary organoleptic criteria used for cultivar selection. If a taxonomic link were established, this could ultimately lead to the incorporation of flavor profile or composition in this selection process. In addition, it was hoped that blueberry flavor compounds would be identified in this investigation that could be used in the formulation of authentic wild blueberry flavors.

In order to process the large number of blueberry samples required for this study, a simple and reproducible isolation method was required. A dynamic headspace gas chromatography- mass spectrometry method was developed that provided these analytical criteria.

Experimental

Blueberry Samples. The mature wild blueberries were cultivated from taxonomically identified plants at the Rutgers Blueberry and Cranberry Research Center, manually collected, and stored at -70°C until assayed. The berries were thawed at 4°C for 24 hr prior to juicing. A total of twenty-three samples comprised of seven wild blueberry species and one mutant albino species (Sooy's) were used in this study, as outlined in Table I. Sample sizes ranged from 200 - 500g total berry weight, averaging 54% yield after juicing.

Table I. Blueberry species evaluated

Sample No.	Vaccinium species	Population Id.	Population Origin
1	*V. boreale*	NJ88-29	Cape Breton Co., Nova Scotia
2	*V. corymbosum*	NC79-76B	Montgomery Co., North Carolina
3	*V. corymbosum*	NC84-6B	Lake Co., Florida
4	*V. corymbosum*	NJ88-8	Highlands Co., Florida
5	*V. corymbosum*	OPB-5	Burlington Co., New Jersey
6	*V. corymbosum*	OPB-8B	Burlington Co., New Jersey
7	*V. corymbosum*	OPB-19	Burlington Co., New Jersey
8	*V. corymbosum*	Sooy's	Burlington Co., New Jersey
9	*V. darrowi*	NJ88-06	Santa Rosa Co., Florida
10	*V. darrowi*	NJ88-12	Liberty Co., Florida
11	*V. darrowi*	NJ88-13	Liberty Co., Florida
12	*V. darrowi*	NJ88-17	George Co., Florida
13	*V. elliottii*	NJ88-01	Bibb Co., Georgia
14	*V. elliottii*	NJ88-03	Jackson Co., Florida
15	*V. myrtilloides*	NJ88-25	Cayuga Co., New York
16	*V. myrtilloides*	NJ88-26	Chippewa Co., Michigan
17	*V. myrtilloides*	NJ88-27	Bayfield Co., Wisconsin
18	*V. pallidum*	NJ88-20	Sussex Co., Delaware
19	*V. pallidum*	NJ89-10A	Madison Co., Arkansas
20	*V. pallidum*	NJ89-12	Yell Co., Arkansas
21	*V. tenellum*	NC78-8	Lexington Co., South Carolina
22	*V. tenellum*	NC83-9	Bladen Co., North Carolina
23	*V. tenellum*	NJ88-31	Turner Co., Georgia

Preparation of Juice Samples. Blueberries were thawed and macerated in a Waring Blendor at high speed for 3 x 15 sec. A 0.1% pectinase solution (Rohapect RD, from Rhom Tech Inc., Malden, MA) was added to the berries at 0.04% berry weight before blending. The slurries were agitated for 30 min, stored at 4°C for 12 hr and then centrifuged at 4,200 rpm for 15 min. The resultant clarified blueberry juice was decanted and analyzed immediately.

Total Sugar - Total Acid Determination. Previous unpublished amphometric HPLC data indicated that the primary sugars in diploid blueberry juice were glucose and fructose. The molar ratio of glucose to fructose in the 23 samples varied from 0.93 to 1.30. Considering the insignificant variation in sugar composition of the blueberry juices assayed, total sugar content (° Brix) was measured with an Abbe refractometer (Fisher Scientific Inc., Pittsburgh, PA) at 20°C. The average of three determinations was recorded with acceptable standard deviations of ≤ 0.2.

Two acid determinations, pH and titratable acidity (TA), were performed on all prepared blueberry juice samples. The pH of the juice was measured using an Orion 620 pH meter (Orion Research Inc., Boston, MA). A 10 g sample of the

juice was titrated with 0.1 N NaOH to a citric acid endpoint (8.33) using a DL-12 Autotitrator (Mettler-Toledo, Inc., Hightstown, NJ). The sugar - acid ratio was calculated as the ratio of total sugar (°Brix) to TA (% citric acid).

Dynamic Headspace Gas Chromatography/Mass Spectrometry Analysis of Volatiles. A 25 mL aliquot of the depectinized blueberry juice was added to a 25 mL fritless sparge vial. The juice was inoculated with 5uL of an internal standard (methanol solution containing 20 ppm fluorobenzene). The juice was sparged with purified helium gas for 11 min at a flow rate of 60 cc/min at ambient temperature (ca. 24°C). Volatiles were trapped onto a Tenax adsorbent trap and desorbed at 180°C for 3 min using an LSC 2000 Purge & Trap, (Tekmar Company, Cincinnati, OH) . Desorbed volatiles were introduced onto a capillary column after passing through a moisture control module held at 4°C.

Separation of the flavor mixture was accomplished by gas chromatography in a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with a 50 m x 0.53 mm (i.d.) Megabore column chemically bonded with a 3.0-μm film of DB-624 stationary phase (J&W Scientific, Folsom, CA), connected to a 1.0-m x 0.53 mm (i.d.) deactivated fused silica retention gap via a Direct-Connect Megabore connector (Alltech Associates, Deerfield, IL). The capillary column was cryogenically cooled at 10°C for 3 min during trap desorption and then temperature programmed at 4°C/min to a final temperature of 180°C and held for 20 min. Inlet and transfer line temperatures were 180°C and 250°C, respectively. Helium was used as the carrier gas at an average linear velocity (μ) of 40 cm/s (10°C).

Mass spectra were obtained using a Hewlett-Packard Model 5971 mass-selective detector linked to a ChemStation data system. The column effluent was passed through a glass jet separator maintained at 220°C. The mass spectrometer was operated at an ionization potential of 70 eV, electron impact mode, scanning from 29 to 450 m/z in a 1.3-s cycle with an ion source temperature of 160°C.

Chemical identifications were accomplished by comparison of the mass spectra and gas chromatographic retention times of the blueberry headspace volatiles to that of injected authentic reference compounds. Headspace concentration of the flavor compounds was calculated with a 3-point internal standard calibration using the Chem Station and EnviroQuant software (Hewlett-Packard). Reference compounds were diluted with methanol and added to a 12.5 °Brix solution of sucrose and distilled water over the experimental range and analyzed using the above procedure. Quantification of volatiles was based on the area beneath the integrated GC-MS peak of the target ion of individual compounds. Characteristic ions selected for quantification were typically the most abundant fragment ion or the parent ion.

Statistical Analyses. Analysis of variance (ANOVA) was performed using SPSS (SPSS Inc., Chicago, IL) software with population, species (replication) and flavor volatile concentration, and sugar/acid ratio (°Brix/ TA) as sources of variation. Significant data was determined by Fisher's LSD ($p \leq 0.05$) and was analyzed with

Principal Component Analysis (PCA) on the basis of mean data using SAS/STAT (SAS Institute, Cary, NC).

Results and Discussion

Berry size of the wild diploid species was mostly (with the exception of *V. corymbosum*) smaller (pea-shaped) in comparison to commercially available cultivars. With the exception of *V. corymbosum*, juicing of the wild diploid blueberry species resulted in low juice/berry yields, ca. 38% (wt/wt), and often resulted in the formation of a gel within 15 min of juicing. On the average, the addition of the pectinase to the macerated berries increased juice yields to 54%, as summarized in Table II. The effect of the pectinase enzyme on juice headspace flavor profile was examined by comparing the concentration of headspace constituents of a treated juice (pectinase treatment) to that of a control (no treatment) juice. Blueberry species (*V. corymbosum*) was used because it contains an insufficient amount of pectin to form a gel. Results indicated no significant change (< the 8.0% RSD of sample reproducibility) in the flavor constituents of the treated vs. the control sample. Depectinization of the blueberry juice also decreased foam formation during sparging of the sample which permitted higher flow rates, ultimately increasing the concentration of volatiles trapped on the adsorbent matrix.

Total sugar and acid values (pH and TA) are summarized in Table II. Total sugars of the wild diploid blueberry species, as determined by soluble solids, averaged 11.5 °Brix (n=23). This value is slightly lower than the 12.5° Brix standard of identity value for blueberry juice. The less than expected Brix value may be indicative of the wild diploid species or it may be due to a seasonal variation. *V. boreale* (n=1) had the lowest total sugar, 9.3 °Brix, while *V. darrowi* had the highest average (n=4) total sugars, 12.9 °Brix.

The acidity of the wild diploid blueberry species, as determined by pH, averaged 3.2 (n=23) with little variation (Sx=0.3) except *V. myrtilloides*., which averaged 2.7 pH (n=3, Sx=0.1). In contrast, the titratable acidity of the wild blueberry species samples ranged from 0.216-1.091% citric acid, with an average TA of 0.519 (Sx=0.265). Consistent with the pH data, *V. myrtilloides* had the highest citric acid content, averaging (n=3) 0.925 %, followed by *V. corymbosum* averaging (n=7) 0.669 %. *V. tenellum* and *V. darrowi* had the lowest amount of citric acid averaging 0.248% and 0.255%, respectively.

The total amount of sugar and the ratio of total sugars to total acids are crucial to the overall flavor quality of a fruit. As a fruit ripens, the total sugars increase which results in an increase in soluble solids. Total sugars and sugar-acid ratio are the primary criteria for the assessment of citrus fruit juices for determination of degree of fruit maturity and overall organoleptic acceptability (10). Variation in sugar-acid ratios of the wild diploid blueberry species was due primarily to variation in acid content of the juice. Juice from *V. myrtilloides* had the lowest average sugar-acid ratio (11.6), while the *V. darrowi* and *V. tenellum* had highest average sugar-acid ratios, 50.6 and 44.9, respectively.

Table II. Sugar-acid determinations of the juice of the wild diploid blueberry species

Sample No.	Species	Population Id.	Yield[a]	°Brix (20°C)	pH	T.A.[b]	Sugar-Acid[c]
1	Vb	NJ88-29	61.5	9.3	3.2	0.483	19.3
2	Vc	NC79-76B	55.4	9.6	3.1	0.623	15.4
3	Vc	NC84-6B	53.6	12.8	3.5	0.504	25.4
4	Vc	NJ88-8	45.6	10.1	3.3	0.646	15.6
5	Vc	OPB-5	55.4	13.6	3.1	0.747	18.2
6	Vc	OPB-8B	56.0	13.5	3.0	0.922	14.6
7	Vc	OPB-19	48.9	11.6	3.2	0.815	14.2
8	Vc	Sooy's	62.4	10.4	3.2	0.427	24.4
9	Vd	NJ88-06	58.2	12.5	3.4	0.278	45.0
10	Vd	NJ88-12	46.7	14.3	3.4	0.269	53.1
11	Vd	NJ88-13	52.2	11.8	3.7	0.222	53.1
12	Vd	NJ88-17	56.6	12.8	3.5	0.249	51.3
13	Ve	NJ88-01	47.8	12.2	3.6	0.402	30.4
14	Ve	NJ88-03	53.0	11.5	3.1	0.750	15.3
15	Vm	NJ88-25	60.2	10.6	2.8	0.757	14.0
16	Vm	NJ88--26	53.4	10.7	2.7	1.091	9.8
17	Vm	NJ88-27	55.1	10.3	2.7	0.928	11.1
18	Vp	NJ88-20	40.8	13.2	3.2	0.445	29.7
19	Vp	NJ89-10A	58.4	11.9	3.3	0.300	39.7
20	Vp	NJ89-12	60.2	9.4	3.3	0.349	26.9
21	Vt	NC78-8	60.2	12.0	3.4	0.247	48.5
22	Vt	NC83-9	50.2	10.0	3.7	0.216	46.2
23	Vt	NJ88-31	55.7	11.2	3.4	0.281	39.9

[a] calculated as % juice wt/berry wt
[b] calculated as % citric acid (wt/wt),
[c] calculated as °Brix/T.A.

A reproducible and simple headspace method was developed to study the genetic variation of wild diploid blueberry species in relation to their characteristic flavor constituents. Due to the nature of the technique, dynamic headspace concentration favors the isolation of the more volatile flavor components. Thus, a number of less volatile components which are present in the juice will inevitably go undetected by headspace analysis. To validate the significance of the blueberry constituents isolated by this technique, several synthetic preparations of the constituents found in Table III were formulated and found to be organoleptically reminiscent of fresh blueberries.

A cursory examination of the total ion chromatograms (TIC) of all the species analyzed in this study indicated similar volatile flavor profiles of samples within the same species. The TIC patterns also seemed to differentiate samples among species. An example of differentiation of the wild diploid blueberry species can be seen in Figures 1&2. The TIC of *V. corymbosum* juice headspace (NJ88-8) shows few volatiles while the TIC of *V. tenellum* juice headspace (NC83-9) is abundant with volatiles.

A total of 36 volatile flavor compounds including 13 esters, 7 hydrocarbons, 6 aldehydes, 5 alcohols, 3 ketones, a cyclic ether and a sulfur-containing compound were identified in the headspace of 23 juice samples comprising 7 wild diploid blueberry species and are reported by GC elution order in Table III. Analysis of variance was used to determine if any of the 36 flavor compounds identified in the 7 diploid species could be used to differentiate the blueberry progenitor species. A subset of 9 compounds from Table III were statistically significant ($p \geq 0.05$) in concentration among the 7 wild diploid blueberry species.

Table III. Volatile compounds identified in the juice headspace of wild diploid blueberry species

acetaldehyde [1]	ethyl-2-methyl propanoate	myrcene
ethanol	methyl-3-methyl butanoate	ethyl hexanoate
dimethyl sulfide [2]	ethyl butanoate	2-methylpropyl-3-methyl butanoate
acetone	1-hexanal [5]	alpha-terpinene
3-methyl-1-butanol	ethyl-2-methyl butanoate [6]	d-limonene
methyl acetate	ethyl-3-methyl butanoate	para-cymene
2-methyl butanal	t-2-hexenal [7]	1,8-cineole [8]
diacetyl [3]	isopropyl isopentanoate	alpha-pinene
ethyl acetate [4]	1-hexanol	terpinolene
3-methyl-1-butanal	ethyl-3-methyl-2-butenoate	2-nonanone
ethyl propanoate	camphene	linalool [9]
methyl butanoate	beta-pinene	1-decanal

[1-9] mean concentrations are statistically significant among wild diploid species ($p \leq 0.05$)

Three of the 9 significant compounds have also been previously reported as character impact compounds for blueberry or bilberry flavor. Parliment and Kolor (2), and Parliment and Scarpellino (11) noted the organoleptic value of t-2-hexenal, t-2-hexenol and linalool to highbush blueberry flavor. Von Sydow and Anjou (7) suggested that the character impact compounds for bilberry are t-2-hexenol, ethyl-3-methylbutanoate and ethyl-2-methylbutanoate.

Blueberry flavor compounds presented in Table III were organoleptically evaluated in dilutions and in trial formulations and were described by experienced flavor chemists. Organoleptically, trans-2-hexenal at 10-100ppm in water has green-vegetative, fatty characteristics. Ethyl-2-methylbutanoate has been previously identified in bilberry, but is reported for the first time in blueberry. Ethyl-2-methylbutanoate is described as fruity, berry-like at 2-20 ppm in water. The flavor impact of ethyl-2-methylbutanoate was dramatically increased when

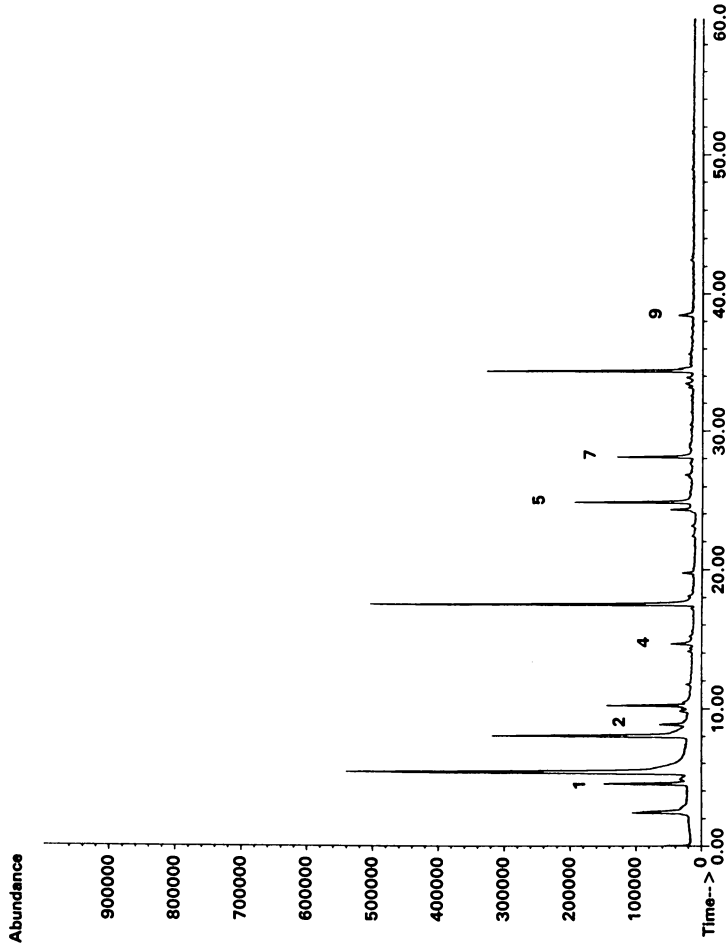

Figure 1. Typical total ion chromatogram of *Vaccinium corymbosum* juice volatiles.

evaluated in an acidified sugar solution (0.05% citric acid, 8°Brix). The importance of linalool to blueberry flavor has been previously described (2). Linalool contributes to the floral, rosey character unique to blueberry fruit at concentrations less than 1 ppm. Concentrations exceeding 2-5 ppm were perfumy.

Other flavor volatiles reported for the first time in blueberry include diacetyl, dimethyl sulfide and methyl acetate. Diacetyl is described as buttery at > 10ppm, however, headspace concentrations ranged from 0.2 to 5 ppm. At low ppm concentrations of diacetyl a ripe character was noted. It is speculated that diacetyl may be produced in the fruit by the presence of lactic acid heterofermentative bacteria. Dimethyl sulfide at 0.1 to 10 ppm has an aroma reminiscent of canned corn and contributes a seedy, ripe quality to the blueberry flavor. Methyl acetate was found at levels from 0.1 to 10 ppm and adds lift to the fruity, floral character of blueberry flavor at these concentrations. These three newly identified compounds are volatile at low temperatures and it is conceivable that previous blueberry flavor isolation methods were incapable of their detection.

Principal component analysis of the 9 compounds produced the eigenvector report shown in Table IV. Eigenvectors 1 and 2 cumulatively represent 69% of the total variance for these 9 compounds, while Eigenvectors 1, 2 and 3 represent 84% of the total variance for these 9 compounds. The two-dimensional PCA plot

Table IV. Eigenvalues of the correlation matrix for nine volatile blueberry compounds.

	Eigen value	Difference	Proportion	Cumulative
PRIN1	3.89	2.28	0.49	0.49
PRIN2	1.62	0.41	0.20	0.69
PRIN3	1.21	0.64	0.15	0.84
PRIN4	0.57	0.24	0.07	0.91
PRIN5	0.33	0.10	0.04	0.95
PRIN6	0.23	0.12	0.03	0.98
PRIN7	0.11	0.07	0.01	0.99
PRIN8	0.04	-----	0.00	1.00

of PRIN2 vs. PRIN1 vectors suggests that the seven species can be differentiated into six groupings (Figure 3): 1)Vb + Ve, 2)Vc, 3)Vd, 4) Vm, 5) Vp and 6) Vt (Table II). The loadings or eigenvectors (Table V) for PRIN1 are all positive, indicating that higher qualities of any volatile would elevate the PRIN1 value. The relatively higher PRIN1 values for Vd and Vt, suggest that these two species contain higher levels of volatiles, overall, than the other species. In addition, Vd and Vt were the only species where 1,8-cineole was detected. For PRIN2, eigenvector values were negative for acetaldehyde, dimethyl sulfide, and

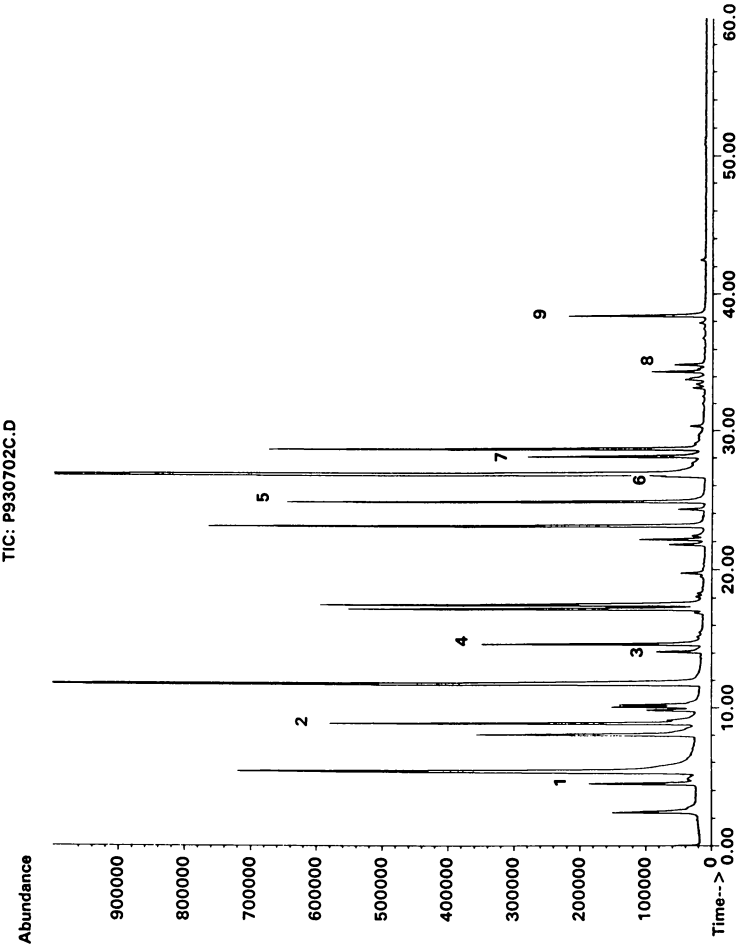

Figure 2. Typical total ion chromatogram of *Vaccinium tenellum* juice volatiles.

linalool, while diacetyl, hexanal, t-2-hexanal and esters were positive. The species Vp which was low in acetaldehyde, dimethyl sulfide and linalool was reflected in Vp having the highest PRIN2 value. One Vt population (NJ88-31) had a low PRIN2 score (-2.3) (Figure 3), resulting from higher levels of linalool and lower levels of diacetyl than the other two Vt populations.

Table V. Principal component analysis: eigenvectors.

	PRIN1	PRIN2	PRIN3
acetaldehyde	0.35	-.40	0.23
dimethyl sulfide	0.18	-.58	0.26
diacetyl	0.11	0.56	0.40
hexanal	0.44	0.23	-.28
t-2-hexenal	0.42	0.21	-.36
1,8-cineol	0.47	0.01	-.16
linalool	0.44	-.15	0.10
esters	0.20	0.26	0.68

Conclusions

The blueberry germplasm consists of 24 blueberry (*Vaccinium* sect. *Cyanococcus*) species, including 7 diploid, 14 tetraploid and 3 hexaploid species (12). The GC-MS analysis of volatiles in the juice headspace of 7 wild diploid progenitor blueberry species indicates species differentiation based on concentrations of a statistically significant subset of aroma compounds. The categorical groupings resulting from the principal component analysis of 9 significant flavor compounds quantified by DH-GC-MS correlate with taxonomic allozyme groupings. The principal component analysis suggests 6 groupings based on fruit flavor volatile profiles. Except for the proximity of *V. boreale* and *V. elliottii*, the remaining species are differentiated from one another. Taxonomically, *V. elliottii* and *V. boreale* are distinct lineages but are similar in their overall volatile profiles. However, they do appear to differ in levels of ethyl acetate and ethyl-2-methyl butanoate. Although the species V. tenellum and V. darrowi are taxonomically recognized as distinct species, allozyme data (1) suggest that they have a closer affinity to each other than with the other diploid blueberry species. Their volatile profiles are consistent with this finding. They can be differentiated, however, based on levels of dimethyl sulfide and ethyl acetate.

Considering that the plant biogenesis of secondary metabolites, including flavor compounds, is enzyme and thus, genetically mediated, varietal flavor differences would be expected. The variation found for fruit flavor volatiles

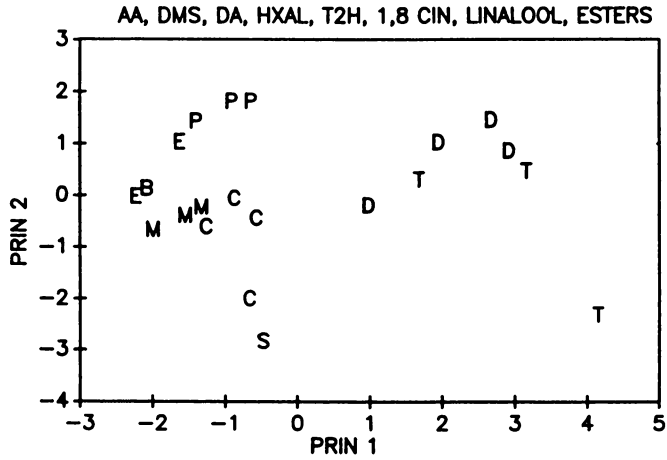

Figure 3. Two-dimensional plot of scores for eigenvector 1 (PRIN 1) vs. eigenvector 2 (PRIN 2) from the 9 significant volatile flavor compounds to categorize wild diploid blueberry species. Sample categories are (B) *V. boreale* , (C) *V. corymbosum*, (D) *V. darrowi*, (E) *V. elliotii*, (M) *V. myrtilloides*, (P) *V. pallidum*, (T) *V. tenellum*, and (S) Sooy's albino.

indicates that in the speciation of blueberry, there was concurrent speciation for fruit chemistry traits. Being that these diploid species are sexually compatible with cultivated germplasm, the opportunity exists for utilizing the diploid species for fruit flavor enhancement of cultivated polyploid varieties. The ability to quantify fruit flavor compounds would facilitate the ability to breed for fruit flavor traits.

Acknowledgments

Gratitude is expressed to Elana Sokol for her assistance in preparation of the juice samples and to Brian Mullin and Steve Ruocco for their organoleptic evaluations.

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Chapter 22

Key Aroma Compounds in Melons

Their Development and Cultivar Dependence

S. Grant Wyllie[1], David N. Leach[1], Youming Wang[1], and Robert L. Shewfelt[2]

[1]Centre for Biostructural and Biomolecular Research, University of Western Sydney, Hawkesbury, Richmond, New South Wales 2753, Australia
[2]Department of Food Science and Technology, Georgia Station, University of Georgia, Griffin, GA 30323

Fruits from most *C. mello* var. reticulatis cultivars typically exhibit a strong and characteristic aroma which is often used by the consumer as an indicator of quality. The odor significance of the aroma components from a range of melon cultivars has been assessed by GC sniffing and aroma dilution techniques. The identification of the key compounds considered to be important contributors to the aroma of high quality melons and descriptors of their aroma character is presented. Many of these aroma compounds are formed during the intense biosynthetic process of ripening from free amino acids in the fruit. The changes in the individual free amino acids, sugars, organic acids and volatiles during the development of the fruit have been determined and possible relationships examined.

Sweetness and aroma are known to be among the most important quality indicators of melon fruit for consumers. To further our understanding of the factors that determine these parameters the changes in the concentrations of sugars, aroma volatiles, free amino acids and organic acids during the development and ripening of Cucumis melo cv Makdimon fruit have been determined. The increase in sugar concentration during ripening is due to the rapid accumulation of sucrose, a process which can only proceed if the fruit remains attached to the plant. Some amino acids known to be precursors to the aroma volatiles show significant increases in concentration during ripening. This increase correlates with that of total volatiles production. In contrast to sugar production, aroma production continues after harvest but sensory evaluation by gas chromatography olfaction methods shows that the aroma profile of fruit harvested only three or four days before full maturity is significantly different from that of fruit harvested at full maturity. Thus for this type of melon the time window available to a producer to harvest fruit having adequate quality and shelf life is quite small.

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There is a large body of evidence that suggests that for many fruits, those ripened on the plant have superior flavour and eating qualities than those harvested before the fully ripened state is reached. However in many cases fruit ripened in this way have a reduced shelf life. Growers are faced with the dilemma of when to pick their crop to obtain maximum eating quality and yet obtain a product which will adequately withstand subsequent handling and distribution environments. Different fruits vary greatly in their ability to meet these two competing criteria. Melons particularly those of the type *Cucumis melo* var reticulatis, commonly known as rockmelon, muskmelon or cantaloupe are a fruit in which this conflict is particularly sharply delineated.

The factors that determine the consumer acceptance of melons have been the subject of a number of investigations *(1-4)* but as yet there seems to be no general agreement on the ones that are reliable indicators of overall fruit quality. Final sugar concentration or sweetness is however certainly one of the major determinants of melon quality. Unlike some fruits, melons have no starch reserve to act as a sugar source during ripening. The large increase in the sugar level that occurs over the ripening period is achieved by the rapid importation of carbohydrates into the fruit from the plant*(5)*. This importation process can take place effectively only if the plant and the fruit remain attached and the leaf structures are both adequate and biosynthetically active; conditions which may not be met if they are diseased or damaged in some way. Thus to obtain desirable sugar levels melons should be picked when very close to the fully ripe condition. However if picked in this condition the resulting fruit may have a shelf life of only a further four to eight days depending on subsequent handling conditions.

On the other hand the factors governing aroma production, one of the other important consumer quality parameters, are largely unknown. Therefore as part of our ongoing investigation of the chemical and biochemical changes in melons and their relationship to fruit quality a detailed study of the development of aroma and aroma precursors during fruit development has been undertaken. At the same time other data on changes in the content and composition of sugars, organic acids and a range of minerals has been obtained.

Some limited radioactive tracer work with melons*(7)* and more extensive work with other fruit such as bananas and strawberries*(8)* which have similar aroma constituents to melons, show that amino acids are the precursors of many of the aroma volatile compounds formed during ripening. The structural skeletons of many of the melon volatiles can clearly be derived from amino acids by a series of well documented biochemical transformations (Figure 1). For example valine can be converted to a range of esters containing the 2-methylpropyl structure, isoleucine to those containing the 2-methylbutyl structure, leucine to the 3-methylbutyl structure and methionine to the thioether group of esters recently identified*(9-13)*. Alanine is also of considerable interest since this compound, when subjected to the same series of transformations, can conceivably supply both the ethyl group and the acetate group found in many of the aroma volatiles. Thus a very large proportion of the compounds that constitute the total melon aroma profile contain structural elements which could be derived from valine, isoleucine, methionine and alanine. It could therefore be expected that aroma development during ripening may depend on the types and concentrations of amino acids available for biosynthesis in the fruit at this time. These, in turn, may depend on the variety or cultivar of the particular melon or on the husbandry practises or conditions to which the plants were exposed.

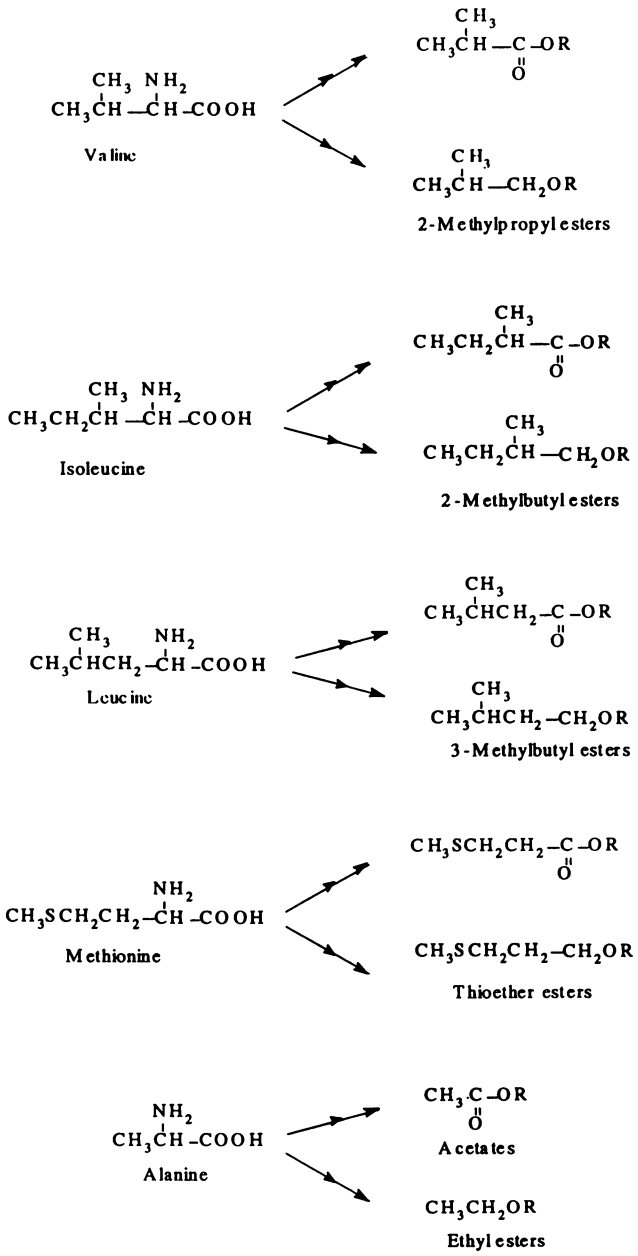

Figure 1. Pathways for the conversion of some amino acids to volatile esters.

Experimental Procedures.

Melons used in this study were from authenticated seed, cv Makdimon, obtained from commercial seed producers. They were grown in a weather protected environment using a hydroponic system with a controlled nutrient mix and therefore all the melons were subjected to the same nutrient and growing regimes. Flowers were tagged on the day of anthesis with inspections being carried out every day during the most intense flowering periods. Three fruit from each age group were selected, stored at 4C and extracted within 24 h. Melon samples were taken from the whole fruit by cutting it into longitudinal sections, the edible portion (middle-mesocarp) removed and cut into small pieces (5x5 cm).

For GC olfactory work samples prepared as above were subjected to simultaneous distillation-extraction (SDE) for 1.5 h using pentane as the extracting solvent. The extract was concentrated (1 mL) in a Kuderna-Danish flask attached to a Snyder column using a bath temperature of 45C. For the GC effluent sniffing experiments(GCO) the concentrated extracts were chromatographed using a Pye Unicam GCV. The outlet from a nonpolar column (J&W DB1, 30 m x 0.32 mm i.d., 1.0μm film thickness) was divided 1:1 using an outlet splitter (SGE,Australia) with one arm connected to an FID detector and the other to a sniffing port (SGE,Australia) flushed with humidified air at 500 mL/min. Chromatographic conditions were: initial temperature: 60C; initial time: 2 min; program rate: 4C/min; final temperature: 200C; injector temperature: 220C; detector temperature: 220C; carrier gas: N_2 at 10 psi. The sensory response to the column effluent was recorded as outlined by Miranda-Lopez et al. *(14)* except that the response was recorded in parallel with the FID using the second channel of a computing integrator (DAPA Scientific Pty. Ltd., Perth, Australia). The resultant aromagram contains a series of peaks which record the intensity and time of the response. Descriptors of each response were recorded during each run on a tape recorder that was subsequently synchronised with the aromagram.

The amino acids after extraction and ion exchange cleanup following the procedure of MacKenzie and Holme*(15)* were analysed by gas chromatography in the form of their TBDMS derivatives using cycloleucine as an internal standard*(16)*.

Sugars and organic acids were converted to their trimethyl silylethers and determined simultaneously by gas chromatography using the method of Chapman and Senter*(17)*.

Volatiles composition and concentration in the samples of melon flesh were determined using a headspace sampler (HP 19393) coupled to a gas chromatograph. Approximately 5g of flesh was obtained by using a cork borer to remove a number of cores from around the equator of the fruit. This was immediately sealed in a headspace vial, placed in the headspace sampler at a bath temperature of 100C, equilibrated for 15min before analysis on an HP 5890 GC fitted with a 30m x 0.32mm i.d. OV1 fused silica column.

Results and discussion.

Carbohydrates. The changes in the concentration of the total sugars with time in the developing melon fruit are shown in Figure 2. This plot shows the typical sigmoidal increase exhibited by melon fruit during ripening*(2)*.

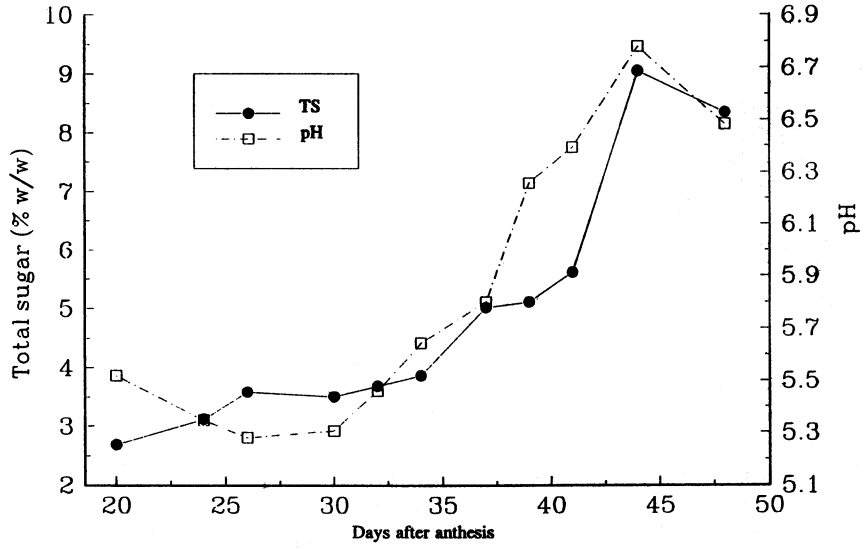

Figure 2. Changes in the Total Sugars and pH during fruit development.

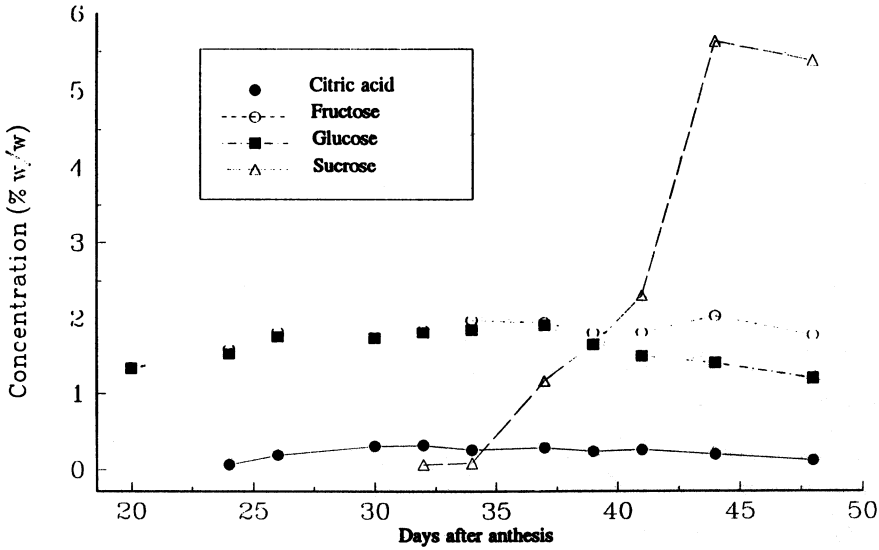

Figure 3. Changes in glucose, fructose, sucrose and citric acid during fruit development.

The onset of ripening is indicated by an increase in total sugars beginning at thirty days after anthesis. This is followed by a rapid rise in carbohydrate concentration over the next five to six days until a maximum is reached on day forty four. Also plotted in this figure are the pH changes that occur in the fruit during development. These two curves are strongly correlated and pose some questions about, for example, the pH dependence of the enzymes involved in the biochemical changes associated with ripening, particularly those involved in the switch from sucrose degradation to sucrose synthesis.

The changes in the concentrations of the three major saccharides of melon fruit, glucose, fructose and sucrose during development are shown in Figure 3. The increase in total sugars seen during the ripening process can be seen to be entirely due to the accumulation of sucrose, the concentrations of glucose and fructose remain essentially constant over the life of the fruit. This is in agreement with the results reported by McCollum et al.*(18)* for a closely related cultivar. The pH changes observed during ripening are not due to changes in the concentration of citric acid, the only significant organic acid present in melons since this showed little variation over the life of the fruit(Fig. 3).

Volatiles. Changes in the total volatiles concentration during fruit development are shown in Figure 4. Very few volatiles were detected in the unripe melon. In the ripening process volatiles production appears to lag sugar production by five to six days but shows a very rapid increase thereafter that maximises simultaneously with sugar production. The changes in the concentrations of the predicted precursor amino acids valine, isoleucine, leucine, methionine and alanine are shown in figures 5 and 6. All of these amino acids show considerable increases in concentration that coincide with the ripening process. Valine, leucine and isoleucine all show increases commencing about thirty five days after anthesis, the same day that the volatiles also begin to make an appearance. Methionine which is also the precursor of ethylene, the ripening hormone, shows a steady increase between day thirty and the attainment of maturity. The increase in alanine concentration lags behind that of the other amino acids by six or seven days but then undergoes very rapid growth to become one of the most abundant amino acids in the mature fruit. This may be because its production is controlled via a different biochemical pathway or because alanine through its conversion to acetyl coenzyme and/or ethanol provides so many of the ester substrates that it begins to rise only when ester biogenesis is slowing down. In the aroma profile of cv Makdimon about twenty four percent of the volatiles contain the ethyl group, forty five percent the acetate group(both able to be derived from alanine), eighteen percent the 2-methylbutyl group (from isoleucine) and ten percent the 2-methyl propyl group(from valine).

The importance of these amino acids as aroma precursors can therefore be appreciated.

Aroma Development. The time course of volatiles production lags that of sugar production by several days. On the other hand the generation of volatiles coincides with the increasing concentrations of certain amino acids some of which are clearly aroma precursors. The increases of these amino acids could occur by increased translocation from the plant or by production in some pathway within the fruit itself or by some combination of both. Our evidence, together with that

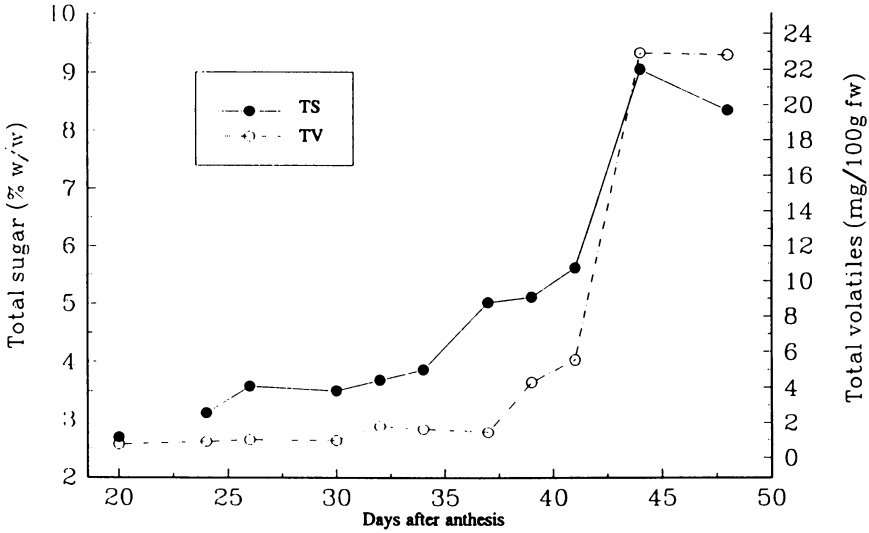

Figure 4. Changes in Total Sugars and Total Volatiles during fruit development.

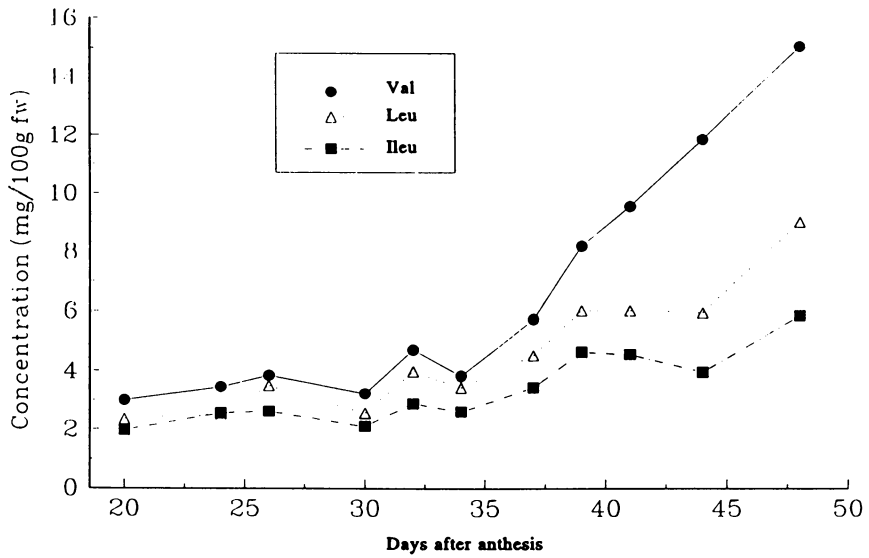

Figure 5. Changes in the concentrations of valine, leucine and isoleucine during fruit development.

of other workers*(6,19)* suggests that the aroma development continues after the fruit is separated from the plant and hence at least the latter of these possibilities is operating. Our preliminary work does however indicate that the aroma development of the separated fruit does not proceed in the same manner as that of a vine ripened fruit. The aroma profiles of melons harvested at half slip and stored at room temperature for three days(i.e. till the predicted time of full slip) were compared with those harvested at full slip and analysed immediately. This comparison was carried out utilising both conventional gas chromatography and gas chromatographic olfactory analysis. The gas chromatographic comparison revealed that the half slip material had only about half of the total volatiles concentration of the full slip material. The traces from the aromagrams are shown in Figure 7. The much greater complexity of the full slip material is immediately obvious. This could be due to two factors. Firstly the greater concentration of the full slip extract means that more of the constituents exceed their odour threshold and hence make a contribution to the aroma profile. Secondly, compounds that appear or increase their concentrations later in the ripening process may now change the aroma profile.

The quantitative importance of the volatiles derived from branched chain amino acids in the aroma extract of cv Makdimon has been referred to above. The sensory importance of these volatiles to the aroma profile has been determined by GCO and aroma extract dilution analysis*(20)*. These techniques show that ethyl 2-methylbutanoate(from isoleucine) and methyl 2-methylpropanoate (from valine) are among the most significant contributors to this melon's aroma profile. These two compounds were also identified as the most potent odorants in an unspecified muskmelon using aroma extract dilution analysis*(21)*. Significantly it is the concentrations of compounds such as ethyl 2-methylbutanoate, ethyl 2-methylpropanoate, 2-methylbutylacetate, 2-methyl propylacetate and the thioether esters that vary markedly from variety to variety and cultivar to cultivar*(10,13,22)*. This suggests that the conversion of amino acids to esters involves enzyme specific pathways which differ between melon varieties and cultivars and hence control their characteristic sensory profiles. Comparison of our amino acid analyses of a highly aromatic melon such as cv Makdimon with those of a melon of low volatiles content such as cv Alice shows that the concentrations of free amino acids found in these widely different melons do not vary significantly. The difference in volatiles concentration therefore is not due to different availabilities of amino acid substrate and must lie elsewhere in the biogenetic pathway.

Fruit Quality Considerations. Examination of Figure 4 shows that if fruit of adequate consumption quality i.e. having at least nine to ten percent total sugars and high aroma values and a reasonable shelf life is to be obtained, the harvest time window is quite small, no more than two to three days before the fully ripe stage is reached. For this judgement to be reliably made the producer needs a sensitive and field portable indicator of fruit development. The gas sensing device reported by Miles et al.*(23)* and the near infrared device described by Dull*(24)* appear to show promise in this area. A well controlled and rapid fruit distribution system from grower to consumer will also be required.

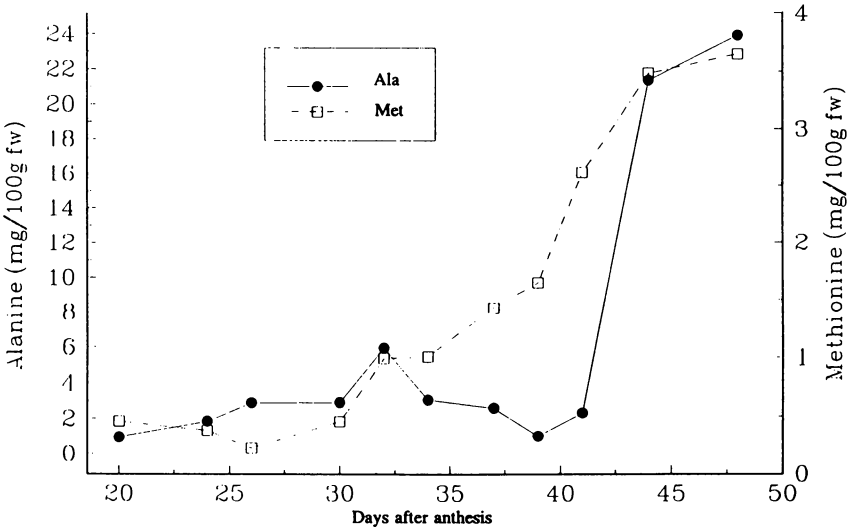

Figure 6. Changes in the concentration of alanine and methionine during fruit development.

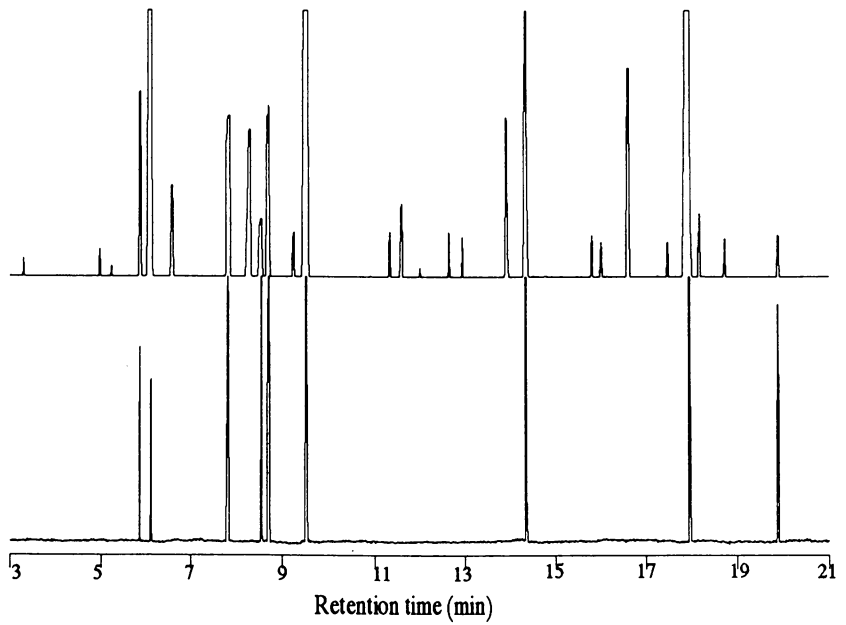

Figure 7. GCO aromagrams of fruit harvested at full slip (top) and stored for 3 days (bottom).

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Chapter 23

Chiral γ-Lactones, Key Compounds to Apricot Flavor

Sensory Evaluation, Quantification, and Chirospecific Analysis in Different Varieties

Elisabeth Guichard

Institut National de la Recherche Agronomique, Laboratoire de Recherches sur les Arômes, 17 rue Sully, 21034 Dijon Cédex, France

Volatile compounds of six apricot cultivars were isolated by vacuum distillation and separated by gas chromatography. Eleven aromatic compounds were selected by sniffing and quantified after addition of known amounts of pure compounds. Sensory analyses were performed to rate the cultivars' apricot aroma. Statistical analyses were performed to correlate the typical sensory notes with the instrumental data. A correspondence analysis showed that cultivars could be separated into three clusters, according to their typical aroma. Hexyl acetate, γ-octalactone, and γ-decalactone were shown to be key compounds for the apricot flavor, whereas benzaldehyde can have a negative or positive impact. Chirospecific analysis of the γ-lactones was achieved by multidimensional gas chromatography. For all the γ-lactones from C_6 to C_{12}, the (R) enantiomer predominated (>90%).

γ-Lactones possess an intense aroma, often described as fruity, peach or coconut-like (1,2). A sensory evaluation of racemic γ-lactones by a panel, using odor profiling, showed that an apricot note was attributed to the γ-octa- and γ-decalactone (3). Tang and Jennings (4,5) were the first to identify γ-lactones, among other volatile compounds, in apricots belonging to the variety Blemheim (in California). These compounds were later related to the pleasant aroma of the French cultivar Rouge du Roussillon by Rodriguez et al. (6) and Chairotte et al. (7).

In the present study, we wanted to determine first the impact of γ-lactones on the typicality of the apricot aroma and then to assess if the chirality of the molecule influences the odor of the lactones.

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MATERIAL AND METHODS

Chemical analyses

Extraction of volatile compounds Volatile compounds of 6 apricot cultivars, which exhibited different sensory characteristics (Table 1), were isolated by vacuum distillation as previously described by Guichard and Souty (8). Tridecane (1 mg/kg fruit) was added to the pulp before distillation, for quantification. The distillates were extracted with dichloromethane.

Table 1: Characterization of the 6 selected varieties.

Code	Cultivar	Size	Firmness	Colour	Taste Quality
PT	Precoce de thyrinthe	Big	Very good	Orange with red surprints	Ordinary
PA	Palsteyn	Average to big	Good	Intense orange with red spots	Ordinary
MO	Moniqui	Very big	Average	Cream with a red part	Excellent, sweet
RR	Rouge du Roussillon	Average or small	Good	Orange with a red part	Very good but pasty
PO	Polonais	Average	Good	Clear orange with some red spots	Good
BE	Bergeron	Big to very big	very good	Orange with red spots	Middle, little juice

Quantification by Gas Chromatography (GC) analysis The concentrated extracts were submitted to GC analysis on a DB5 fused silica capillary column (30 m, 0.32 mm i.d., 1 mm, J. & W. Sci.).

The odor of the individual substances eluted from the column were assessed separately using the device described by Etievant et al. (9). Eleven aromatic compounds were selected by sniffing the GC effluent and quantified after addition of known amounts of pure compounds (10).

Chirality evaluation by multidimensional GC analysis Determination of the enantiomeric ratios of the γ-lactones in apricots was achieved using multidimensional GC on a Siemens Sichromat 2-8 with a DB 1701 fused silica capillary column coupled to a heptakis (3-O-acetyl-2,6-di-O-pentyl)-β-cyclodextrin column, as already described by Guichard et al. (11).

Sensory analyses

Evaluation of cultivar characteristics Sixteen subjects were asked to smell each sample and to rate on an unstructured scale (from 0 = non typical, to 100 = very typical), how close the odor was from their internal reference of a typical fresh apricot.

Determination of odor thresholds and odor quantities of optically pure compounds Thresholds were determined on each optically pure enantiomer by a 1/3 test, by ascending concentrations. Individual thresholds were calculated according to Meilgaard and Reid (12). Iso-intensitive concentrations between each enantiomer and the racemic corresponding lactone were determined by positioning two different concentrations of each enantiomer on a scale represented by five ascending concentrations of the racemate. Each panelist had then to describe the perceived odor, using the lists of descriptors previously determined on the racemic compounds (Table 2), and to attribute a note of intensity between 0 and 4 for each descriptor.

Table 2: Descriptors used for each lactone.

γ-octalactone	γ-decalactone
fruity	fruity
peach	peach
coconut	coconut
apple	apple
mint	mirabelle plum
tobacco	apricot
dust	pear
detergent	canned fruits
	strawberry

Statistical analyses

Correspondence analysis (CA) was used to evaluate the chemical data (6 lines = cultivars, 11 columns = quantity of each compound) with typicality notes (TY+) and their complement (TY- = 100 - TY+) as descriptive elements (13). As cultivars, compounds and typicality notes were plotted together, correlations between sensory and chemical data could be observed.

RESULTS AND DISCUSSION

Characteristics of apricot aroma. Preliminary results showed that there was no sensory detectable difference in the odor typicality of the distillate and that of the

apricot pulp. Correlations could thus be established between sensory analyses made on the pulp and chemical analyses realized on the distillate.

The concentrations of the 11 selected aromatic compounds (Table 3) arranged according to increasing scores of odor typicality are presented in Fig. 1a and 1b. As 2 clusters of cultivars were revealed by multiple comparison, a

Table 3: Codes, names and odor descriptions of volatile compounds (obtained from ref 3.)

Code	Name of compound	Odor description
ACHE	hexyl acetate	fruity, banana, pear
GOCL	γ-octalactone	fruity, coconut
C3HL	cis-3-hexenol	floral, herbaceous
ACBU	butyl acetate	floral, banana, fruity, nail polish remover
GHEL	γ-hexalactone	fruity, floral
DOCL	δ-octalactone	fruity, apricot
C3HA	cis-3-hexenyl acetate	green, herbaceous
2HON	2-heptanone	fruity, almond
LINA	linalool	floral, orange flower
GDEL	γ-decalactone	fruity, peach
BZAL	benzaldehyde	almond

Students' t test was performed for each compound between these 2 clusters. Mean notes of typicality and corresponding t values are given on the same figures. The varieties "Precoce de Thyrinthe" and "Palsteyn" have been assessed as less aromatic, which was in agreement with the results of the instrumental analyses showing that the flavor impact compounds are present in very low amount. The results of CA on chemical compounds and cultivars are shown in Fig. 2. As the assessors did not evaluated the typicality of the odor in the same way, they were divided into 3 clusters defined in Table 4 (13). The scores of typicality for each cluster of assessors are plotted as descriptive elements. Hexyl acetate, γ-octalactone and γ-decalactone were shown to be highly correlated with the

Table 4: Means of the sensory scores for each cluster of subjects.

	CULTIVARS					
Cluster	PT	PA	MO	RR	PO	BE
N° 1	5.5	14	50	52	81	67
N° 2	9.0	5	68	59	50	55
N° 3	0.0	2	82	71	24	71

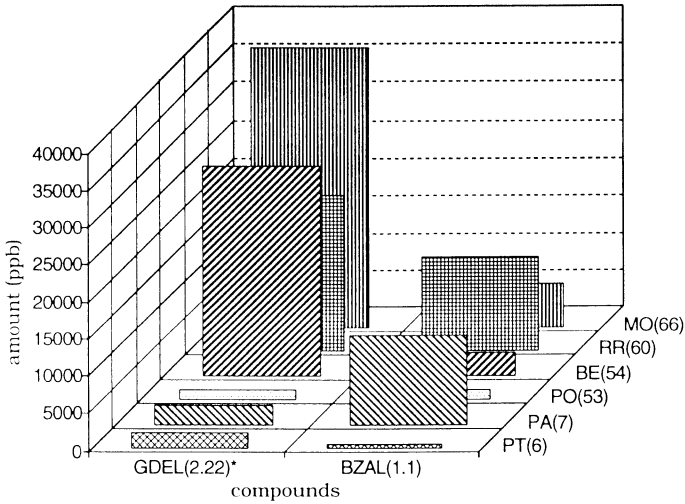

Figure 1a: **Amount of volatile compounds (ppb) in each cultivar of Table 1. For each compound, the values in brakets are the student's t test values between the 2 clusters of cultivars (PA, PT / MO, RR, BE, PT) and the level of signification (*** = 0.1%, ** = 1%, * = 5%), for each cultivar, the values in brakets are the typicality notes.**

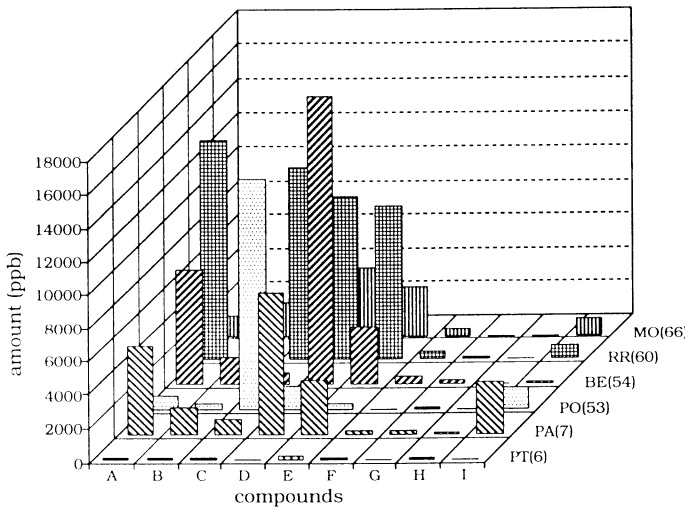

Figure 1b: **A = ACHE(15.42)***, B = GOCL(3.47)**, C = C3HL(2.24)*, D = ACBU(1.98), E = GHEL(1.4), F = DOCL(0.79), G = C3HA(0.71), H = 2HON(0.34), I = LINA(0.22).**

typicality of the apricot flavor, whereas benzaldehyde could have a negative impact on the aroma of the less aromatic cultivars but would favorably complete the typical aroma of "Rouge du Roussillon". "Polonais" has been described by green flavor notes due to the great amounts of aliphatic aldehydes and alcohols, this cultivar was preferred by subjects of cluster 1. "Moniqui" which contained a lot of esters and ketones was described with floral notes and judged as the most typical by subjects of cluster 3, but "Bergeron" and "Rouge du Roussillon", with their high amounts of lactones possess a fruity aroma, and a rather good typicality for all the subjects.

Enantiomeric ratios of lactones in apricots Lactones possess an asymmetric carbon atom and thus 2 optical isomers exist which are mirror images. In natural products such as fruits, only one enantiomer is found. In apricots we determined that, for the γ-lactones from 6 to 12 carbon atoms, the (R)-enantiomer always predominated (Table 5). It could thus be possible to determine , in products containing apricot, an adulteration by addition of a racemic synthetic lactone.

Table 5: (R)-enantiomer ratios of γ-lactones (from C6 to C12) in the different cultivars.

CULTIVAR	C6	C7	C8	C9	C10	C12
PT	99	81	90	64	91	93
PA	82	80	90	-	92	99
MO	91	81	90	83	95	99
RR	71	90	88	77	98	99
PO	96	81	92	76	95	99
BE	88	89	93	83	96	98

Sensory evaluation of the enantiomers of lactones For each lactone, the individual thresholds distributions are plotted on Fig. 3 with their threshold mean values. Despite a large inter individual variability, the mean value of the (*R*)-γ-octalactone, computed on the whole panel, tends to be higher (but not significant) than that of the (*S*)-γ-octalactone, and most of the subjects (18/28) have a higher detection threshold for the (*R*)-lactone (Fig. 3a). At a concentration 100 times higher than its odor threshold, the (*S*)-enantiomer possess an odor more intense than the (*R*)-enantiomer (Table 6). For the same lactone, Mosandl and Günther (14) found that, at a concentration of 1% in propylene glycol, the odor of the (*R*)-enantiomer was more intense than that of the (*S*)-enantiomer. However, the molecules were nor evaluated by the same panelists neither with the same sensory tests, which could explain the differences in the conclusions. For the γ-decalactone, the mean value of the (*R*)-enantiomer tends to be lower than the

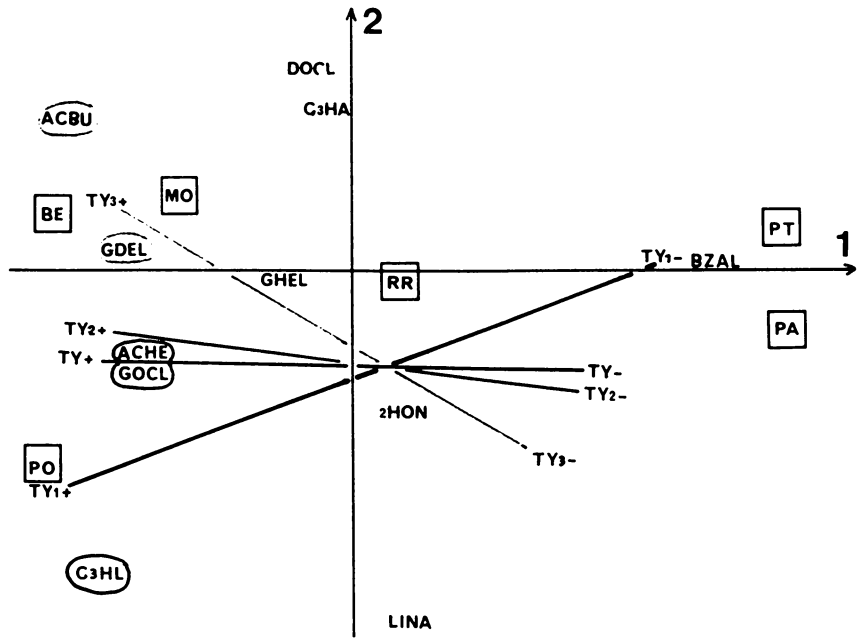

Figure 2: Plane representation of the correspondence analysis of the chemical data. Codes of volatiles refer to table 3. Codes of cultivars refer to table 1. TY+: illustrative projection of typicality note given by the whole panel. TYi+: illustrative projection of typicality note given by the ith cluster of assessors.

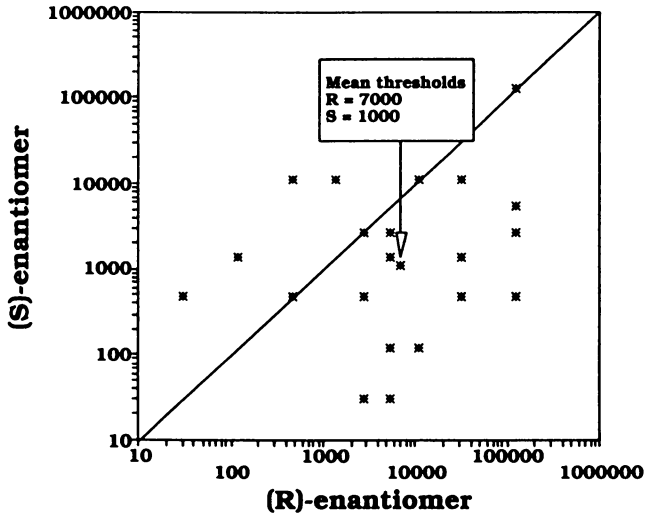

Figure 3a: Individual and mean thresholds of the enantiomers of γ-octalactone (10^{-3} ppb).

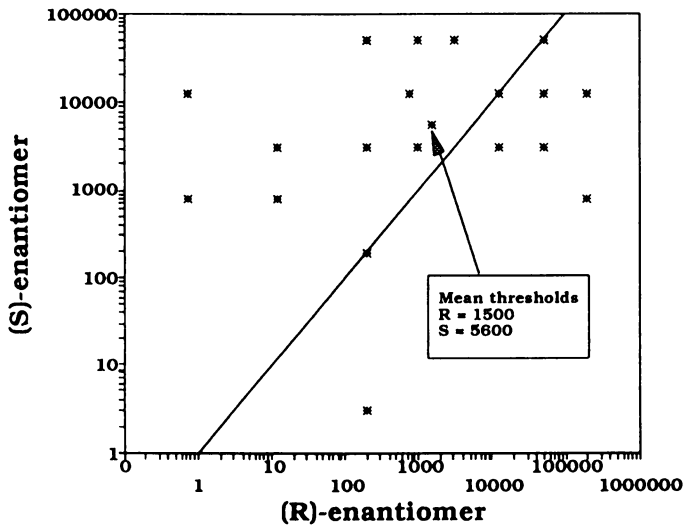

Figure 3b: Individual and mean thresholds of the enantiomers of γ-decalactone (10^{-3} ppb).

mean value of the (*S*)-enantiomer (Fig. 3b) but this last enantiomer shows a higher odorous power at a high concentration (Table 6).

Table 6: Iso-intensitive concentrations (ppm).

	γ-octalactone		γ-decalactone	
racemate	*R*	*S*	*R*	*S*
0.5	1	0.12	1.6	0.4

Concerning the odor assessment at iso-intensitive concentrations, no significant differences were found between the 2 enantiomers for the main odor notes (fruity, peach) and for most of the other descriptors. However, at the same concentration, the (*S*)-γ-octalactone was described as more coconut-like than the (*R*)-enantiomer, which was described as more strawberry-like (Fig. 4). This could suggest, according to the theory advanced by Chastrette et al. (15), that more than one receptor site exists for the perception of these odors and that the chirality center is not involved in the interaction with the receptor site related to the fruity note. Taking into account the iso-intensitive concentrations (R2 and S1), no significant difference was found between the odors of the 2 enantiomers.

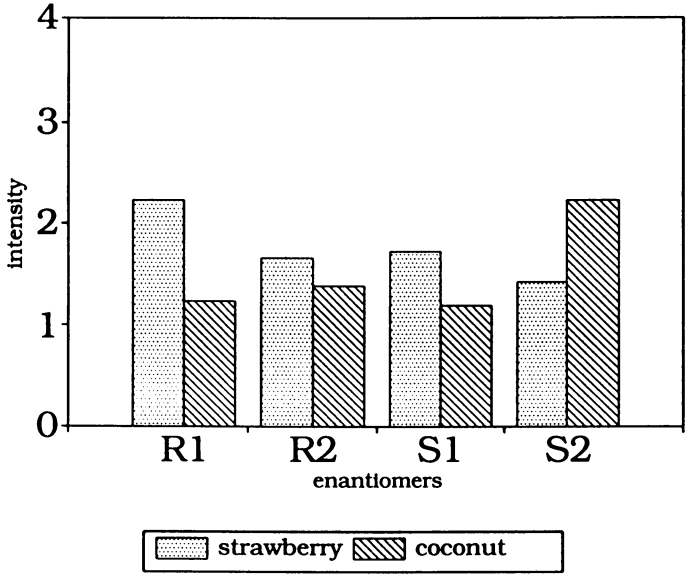

Figure 4: Odor notes with significant differences between the enantiomers of γ-decalactone. (R1 = 0.4 ppm, R2 = 1.6 ppm, S1 = 0.4 ppm, S2 = 1.6 ppm).

Differences in odor quality seem thus to be only due to differences in odor intensity.

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Chapter 24

2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone and Derivatives in Strawberries During Ripening

Carlos Sanz, Daryl G. Richardson, and Ana G. Pérez

Department of Horticulture, Oregon State University, Agriculture and Life Science 4017, Corvallis, OR 97331

Content of Furaneol and derivatives in seven strawberry varieties were assessed during ripening. In most cases content of these compounds sharply increased along fruit ripening with maximum values at the overripe stage. The largest amount of Furaneol, mesifurane and Furaneol glucoside were found in overripe strawberries of cultivars Douglas (22.89 μg/g FW), Pajaro (39.13 μg/g FW), and Totem (16.51 μg/g FW), respectively. Results obtained showed quantitative differences among varieties that could be related to their organoleptic properties. The best correlation values were found between Furaneol content and strawberry aroma for Parker (r=0.741) and Benton (r=0.733) strawberries.

It seems to be a general phenomenon that increase in berry size, obtained by breeding, inevitably leads to deterioration of the aroma of berries. Thus, the pleasant and herbaceous aroma of wild strawberries is not found in most cultivated varieties.

Strawberry aroma is mainly determined by a complex mixture of esters, aldehydes, alcohols and sulfur compounds which have been extensively studied (*1-5*). Two compounds, 2,5-dimethyl-4-hydroxy-3(2H)-furanone (Furaneol, I) (*6-7*) and 2,5-dimethyl-4-methoxy-3(2H)-furanone (mesifurane, II) (*8*) are considered to be among the most important volatiles reported in wild strawberries. These two compounds have not been found in all cultivated varieties (*9*), although factors such as Furaneol water-soluble nature (*8-10*) and thermal unstability (*11-12*) could well account for the failure of some authors to detect Furaneol. Both, Furaneol and mesifurane, have strong, sweet and pleasant odours. Furaneol imparts caramel burnt sugar notes at high concentrations and becomes fruity, strawberry-like at lower concentrations (*13*), and mesifurane is described as having a more sherry-like aroma (*14*). Kallio et al. (*15*) found in artic

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bramble good correlation between Furaneol content and the aroma evaluations, and a certain influence of the content of mesifurane on the overall impression of taste.

There are several studies identifying the presence of Furaneol, mesifurane (*16-17*) and Furaneol glucoside (III) (*18*) in strawberries. However, no study has focused on the production of these three compounds during fruit ripening, and due to the lack of a reliable quantitative method of analysis their actual contribution to strawberry aroma is still not well known.

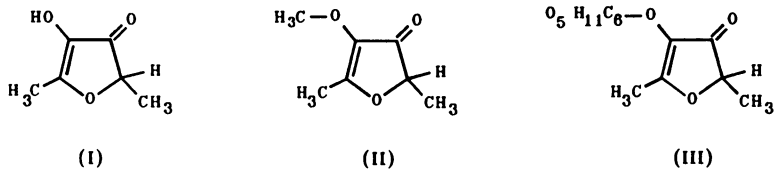
In this work a new analysis procedure, involving HPLC separation and quantitation of Furaneol, Furaneol glucoside and mesifurane, is used to determine the amount of these three compounds in seven strawberry cultivars during ripening. The relation of Furaneol and mesifurane contents to aroma evaluations is also assessed.

Experimental

Fruits. Fruits from seven strawberry (*Fragaria ananassa* Duch.) cultivars, Chandler, Parker, Douglas, Pajaro, Benton, Redcrest and Totem, grown at the O.S.U. horticultural research fields in Corvallis (OR, USA), were used in this study. Strawberries were harvested at four ripening stages: white (I), pinky (II), bright-red (III, ripe) and dark-red fruits (IV, overripe), and immediately frozen, and kept at -25°C.

Preparation of samples for HPLC. Strawberries were cut symmetrically in four pieces. Four pieces from four different fruits, approximately 15 g, were thawed and ground in a Sorvall Omni-mixer with 15 mL of distilled water. Celite (1.5 g) was added and mixed with the homogenate, and allowed it to stand for 5 min. This mixture was vacuum filtered through a Whatman No 1 filter paper (Whatman Int. Ltd., Maidstone, UK), and the solid phase washed three times with 5 mL distilled water. Five mL of this filtered extract was clarified, removing pulp, fat, protein, and carotenoids, by first adding 0.25 mL of Carrez I solution, and then 0.25 mL of Carrez II solution added slowly with gentle mixing, according to Wallrauch (*19*). After standing for 5 min, the mixture was centrifuged at 2500 x g for 5 min. The supernatant was filtered through a 0.2 μm nylon membrane (Alltech Associates, Inc., Deerfield, Il) before HPLC analysis.

HPLC analysis. Quantitative HPLC analysis of strawberry extracts was accomplished with a Beckman 334 liquid chromatograph (Beckman Instruments Inc., Berkeley, CA), Hitachi 100-10 detector (Hitachi Ltd., Tokyo, Japan), and Shimadzu C-R3A integrator (Shimadzu Co., Kyoto, Japan). Analysis was carried out using a reverse phase Econosil C18 column (25 cm x 4.6 mm, 10 μm, Alltech) coupled to a ODS-5S guard column (3.0 cm x 4.6 mm, Bio-Rad, Richmond, CA). The mobile phase consisted of: A) 0.2M sodium acetate/acetic acid (pH 4) buffer (acetate buffer) and B) methanol, with the following chromatographic conditions: 0-2 min, isocratic 10% methanol; 2-18 min, gradient 10-12% methanol; 18-36 min, isocratic 12% methanol; flow rate, starting 1.5 mL/min and increased to 2.0 mL/min in 0.5 min at 18 min; detector, UV 280 nm; and injection volume, 20 μL.



(I) (II) (III)

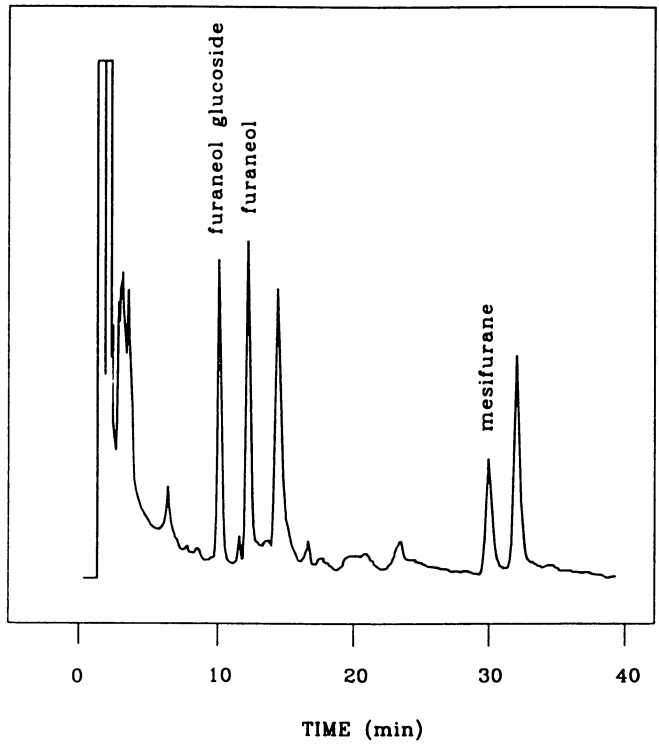


Figure 1.-Typical HPLC chromatogram of a strawberry extract (cv. Totem, stage IV).

Sensory evaluation. Extracts from each strawberry variety at different maturity stages were evaluated by means of a 16-point scale for strawberry aroma and overall aroma intensity. Samples were assessed by 18 judges at room temperature. A random three-digit code was given to each sample, and they were served in a random presentation, either three or four samples at a time. Each sample, 25 mL of strawberry extract, was presented in a 75 mL odorless glass jar covered with black opaque paper. Linear regression coefficients for furaneol and mesifurane contents against strawberry aroma and overall aroma intensity were calculated.

Results and discussion.

HPLC analysis. Gas liquid chromatography is the most frecuently used method for the separation of aroma compounds of strawberry. However, this technique has been proved to be inadequate for the determination of compounds such as Furaneol due to the thermal unstability of this compound under normal GC conditions (*12, 20*). This can explain the low reproducibility of the results found in early reports on Furaneol content in fruits (*7-8, 21*). HPLC analysis seems to be a more suitable technique to determine the actual content of Furaneol, mesifurane and Furaneol glucoside in fruits, as it has been shown in recent studies of Furaneol content on grapefruit and pineapple (*22-23*). Nevertheless no method for the simultaneous determination of the three compounds in strawberry has been reported.

Figure 1 shows a typical HPLC chromatogram from a strawberry extract, where Furaneol glucoside, Furaneol and mesifurane were resolved into unique peaks at retention times of 10.0, 12.2 and 30.3 min, respectively. The analytical method used is faster than traditional methods involving liquid-liquid extraction (*7-8, 21*), and avoids any kind of concentration procedure which could cause alterations in the aroma composition.

Furaneol and Derivatives Content during Ripening. In order to understand the aroma of a fruit is necessary to know not only the nature of constituents, but how the significant components change in kind and quantity during the development of the fruit. Using the analitical procedure described above, Furaneol and derivatives content were determined in seven strawberry varieties during ripening (Figures 2 and 3).

In all studied varieties only when fruits reached a certain degree of ripeness, the biosynthesis of the three compounds was enhanced. Furaneol, Furaneol glucoside and mesifurane content sharply increased at the last ripening stage. These results seem to agree with those reported on the formation of methyl and ethyl esters during strawberry ripening (*5*). However there are other groups of compounds such as amyl, isoamyl and hexyl esters whose contents remain constant or decrease at the last maturity stages, as reported in Chandler strawberries (*5*).

Although the biogenetic pathway of Furaneol is still unknown, the reason for the presence of this compound and derivatives only at the last ripening stages could be the lack of the forming enzyme activity in unripen fruits, as proposed Yamashita et al. (*24*) for volatile esters.

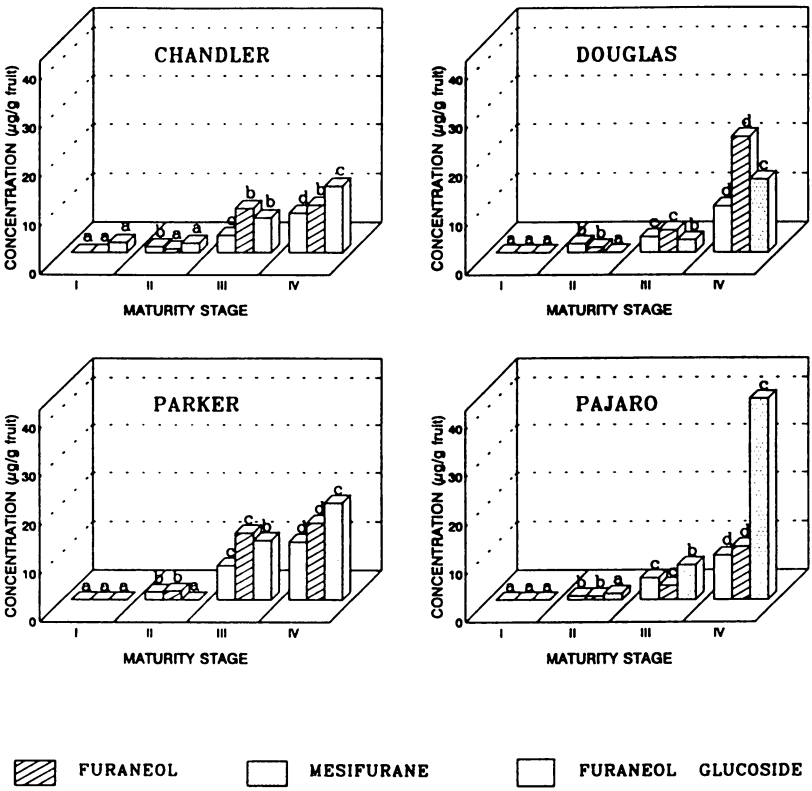


Figure 2.-Content in Furaneol, mesifurane and Furaneol glucoside in Californian varieties during ripening. Each bar represents the mean of six analyses. Means for the same compound with the same letter are not statistically different at significance level p=0.05.

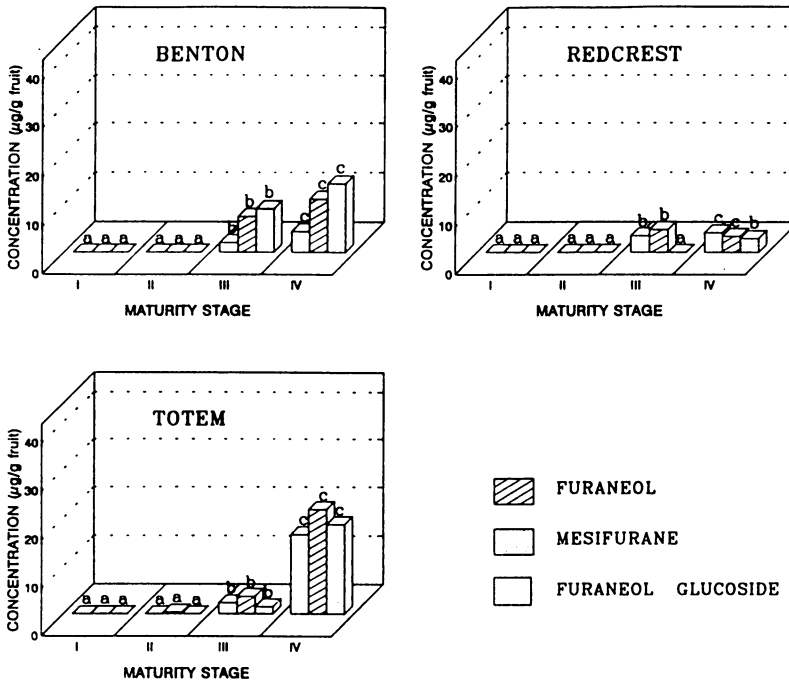


Figure 3.-Content in Furaneol, mesifurane and Furaneol glucoside in strawberry varieties from Oregon (Benton, Redcrest) and British Columbia (Totem) during ripening. Each bar represents the mean of six analyses. Means for the same compound with the same letter are not statistically different at significance level p=0.05.

Among the seven strawberry cultivars studied, four were Californian varieties, Chandler, Parker, Douglas and Pajaro, two were from Oregon , Redcrest and Benton, and Totem was from British Columbia. Most studies, dealing with the quantitative comparison of volatiles, have found great differences among cultivars (*4, 9, 17, 25*). In this work, a quite similar pattern on the formation of the three compounds under investigation was found for all the Californian varieties (Figure 2). In these four strawberry cultivars, low amounts of Furaneol, Furaneol glucoside and mesifurane were found to be present in the fruits in early ripening stages (I and II), while almost total absence of these three compounds was determined for Redcrest, Benton and Totem at the same stages (Figure 3). At the commercial maturity stage (stage III) Parker showed the highest content in the three compounds, and at the overripe stage (IV) the largest amount in Furaneol and mesifurane were determined in Douglas and Pajaro, respectively. In these varieties, the amounts of Furaneol (22.89 μg/g FW) and mesifurane (39.13 μg/g FW) are the greatest values so far reported in strawberries, only comparable to values described for cultivar Confitura by Douillard and Guichard (*9*). These results were expected, since this is the first non-gas chromatographic analysis of these compounds in strawberries. Similar differences are found when HPLC quantitation data of Furaneol in pineapple (*7, 23*) are compared to those obtained by GC analysis (*26*).

Strawberry varieties from Oregon (Redcrest and Benton) and British Columbia (Totem) are mainly used for the processing industry. Redcrest, a strawberry with very poor organoleptic properties, had the lowest levels in Furaneol and derivatives of the seven studied cultivars. Benton is considered to be a high flavor variety, suitable to be used for jam production specially because its high content in organic acids (*27*). Furaneol and mesifurane content in Benton strawberries stage III were among the highest determined, but only a slight increase was observed in the production of these products at stage IV. Low levels of Furaneol glucoside were determined in this cultivar. Strawberries from cultivar Totem were characterized by having excellent organoleptic characteristics at the last ripening stage (IV). Maximum values of Furaneol glucoside were found in this cultivar (16.5 μg/g FW), which also showed large amounts of Furaneol (21.61 μg/g FW) and mesifurane (18.51 μg/g FW). Given these high contents and that Furaneol has a considerably low threshold concentration (0.03 ppb), while mesifurane has a threshold of 0.01 ppm (*28*), the excellent aromatic quality of this strawberry cultivar reported in a previous study (*29*), would be confirmed.

Sensory evaluation. Sensory evaluation data from the four maturity stages for each strawberry variety were pooled in order to carry out regression analysis of Furaneol and mesifurane contents against values for strawberry aroma and overall aroma intensity. In general terms, the content of Furaneol and mesifurane describes better the strawberry aroma than the overall aroma intensity. On the other hand, there are better correlations for Furaneol content and both attributes than for mesifurane, with the exception of Parker (strawberry aroma and overall intensity) and Benton (strawberry aroma). Kallio et al. (*15*) also found better correlations for Furaneol content than for mesifurane against character of odor in the sensory analysis of different varieties of arctic bramble. The best correlation values between Furaneol content and strawberry aroma were found for Parker (r=0.741) and Benton (r=0.733) strawberries.

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Chapter 25

Characterization of the Putrid Aroma Compounds of *Ginkgo biloba* Fruits

Thomas H. Parliment

General Foods USA, Technical Center, 555 South Broadway, Tarrytown, NY 10591

The Ginkgo family of trees is represented by a single surviving species, *Ginkgo biloba*. It is an ancient line unlike any other living conifer and may represent a link between the conifers and the tree-ferns. In the fall female trees produce yellow-orange fruits with a thick fleshy layer surrounding an edible kernel. The odor of these fruits is described as putrid. This study identified the odorous principles as high levels of butanoic and hexanoic acids.

The Ginkgo family (Ginkgoaceae) is represented today by a single surviving species of tree, native to southeastern China. The *Ginkgo biloba* tree has been introduced to North America where it has become valued as a street and park tree. It represents an ancient line of tree that is unlike any other living conifer. Some believe ginkgos are a link between the conifers and the more primitive plants: the tree-ferns and cycads.

Extracts of dried ginkgo leaves have been used therapeutically for centuries. The extract is semipurifed to produce a mixture of ginkgo flavone glycosides and terpenoids (ginkgolides). Ginkgolides have not been found in any other living plant and differ only in number and position of hydroxyl groups. This extract has shown value in curing cerebral and peripheral circulatory disturbances. Symptoms in elderly people said to be relieved by ginkgo treatment include difficulty in concentration, absent mindedness confusion, lack of energy, decreased physical performance, depression, anxiety and headache (1).

Both male and female trees exist. Male trees are preferred because they don't produce fruits as females do and because they can grow in poor, hard-packed soil and polluted air.

In the fall, female trees produce numerous fruits on their branches. (Figure 1). When mature, these circular fruits are yellow to orange in color and about 2.5 cm in diameter. The flesh surrounds a hard, tan shell that contains the green soft edible kernel of the seed. In the Far East, these seeds are roasted and eaten. The flesh of the

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mature fruit gives off an aroma described as "putrid" or "foul" (2). The presence of these fruits is the main reason that female trees are considered undesirable.

The purpose of this paper is to identify the components responsible for the putrid aroma of the ripe fruits and to determine their approximate level.

Experimental

Sample Preparation Ginkgo fruits *(Ginkgo biloba)* were obtained from the Bronx Botanical Gardens, N.Y.C. The fruits were of varying degrees of maturity and were separated into four arbitrary maturity stages.

Four grams of ginkgo flesh were homogenized with 20g of deionized water. The homogenate was indirectly steam distilled and 8 mL aqueous condensate collected. One gram of sodium chloride was added and the system was extracted with 0.8g diethyl ether (containing 1 μL ethyl nonanoate per 10mL diethyl ether) using a Mixxor (Altech, Deerfield, IL) as described by Parliment (3).

Analysis The samples were analyzed on a Perkin Elmer Model 3920 gas chromatograph which was equipped with a DB225 (25% cyanopropyl/25% phenyl/50% methyl silicon) column measuring 30m x 0.53mm i.d. x 1μ thickness. The column was held 4 minutes at 80°C and then programmed to 200°C at 8°C/min. Samples were analyzed in duplicate and data collection and reduction performed on a Perkin Elmer Nelson Model 2600 Data System.

Component identification was confirmed by GC/MS using a Varian Model 3700 interfaced to a Finnigan Ion Trap Mass spectrometer. The sample was separated on a DB5 (phenyl methyl silicon) column measuring 15m x 0.32mm i.d. x 1μ thickness. Spectra of the separated components were compared to spectra generated from pure reference materials.

Results and Discussion

The distillates from the ripe fruits possessed a strong cheesy rancid aroma, as did the ethereal extracts. The extracts were analyzed by GC/MS techniques. A unique aspect of the ginkgo fruit is the fact that it contains essentially only two volatile compounds, butanoic and hexanoic acids. These two components are respectively described by Arctander (4) as "reminiscent of rancid butter" and "fatty-rancid...sweat-like".

The levels of these components at various stages of ripeness are presented in Table I. Also presented in the Table are the reported threshold values of these two compounds.

It is apparent that the unripe fruits do not have measurable butanoic or hexanoic acids and at this stage of maturity we observed them to be without appreciable odor. Upon ripening the fatty acid levels increase dramatically, reaching a combined level of 500 ppm in the ripe orange stage. The levels are significantly above the threshold values of each components, which explains why these fruits have a reputation for a putrid aroma.

The ginkgo nut is roasted and consumed in a number of cultures. We analyzed the nut for presence of the lower fatty acids and were unable to detect any of these compounds. Previous workers (5) have analyzed the fatty acid composition of ginkgo

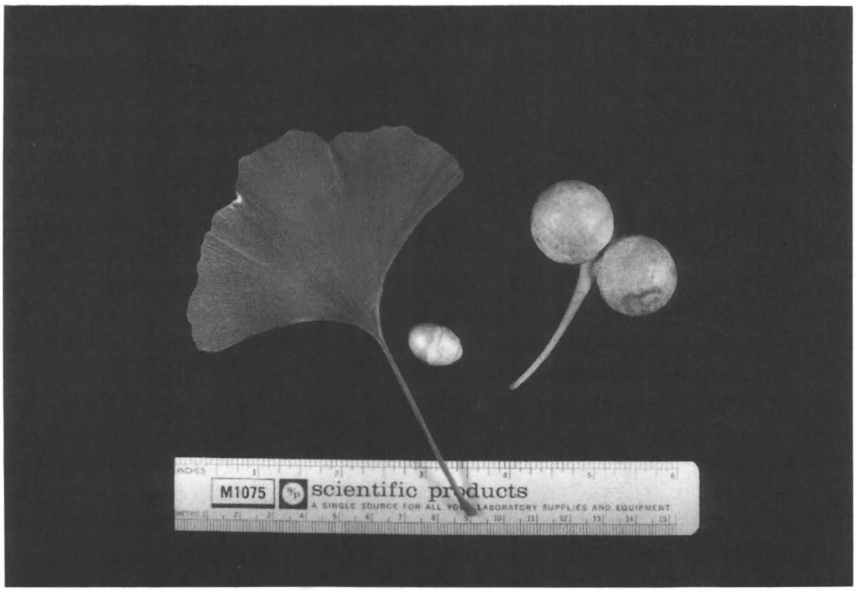

Figure 1. Ginkgo Leaf (left) Kernel (center) and Fruits (right)

nuts and found them to contain 5.1% total lipid and identified the typical C14:0 to C22:1 fatty acids. No acids lower than C14 were detected.

Table I. Free Fatty Acid Levels in Ginkgo Biloba Fruits

MATURITY LEVEL	mg/Kg FLESH	
	n-Butanoic Acid	n-Hexanoic Acid
Green	0	0
Green/Orange	240	90
Yellow/Firm	300	250
Yellow/Overripe	300	200
Kernels	0	0
Thresholds(6)		
Water(mg/Kg)	0.1-1.0	1.0-10.0
Air(mg/m^3)	0.004-0.02	0.07-0.1

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