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Flavor Chemistry

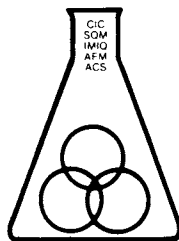
Trends and Developments

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Foreword

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Preface

FOOD IS ONE OF THE MOST INTIMATE AND IMPORTANT COMPONENTS OF our chemical environment. Whether we accept or reject food depends mainly on its flavor. Research into the chemistry of desirable and undesirable foods has become very popular; the advent of modern instrumentation has introduced rapid changes in the field.

The sequence of emphasis in flavor chemistry research has been the following:

1. Experimental methods, or how to obtain the information we need. This aspect must continue as problems being studied become more complex.
2. Correlation of chemical structure to sensory properties. Research into this topic must also be continued. Some simple problems have been solved, and now more complex flavors are being elucidated.
3. Formation origin and mechanisms of flavors. This has always been a topic of interest, but with more definite information available about the chemical composition of characteristic flavors, more definite paths of biological and chemical origin can be postulated and verified.

Understanding the mechanism by which flavor compounds are formed can lead to better methods of food processing for better formation and retention of flavor. Fundamental flavor chemistry information is also essential in genetic engineering of plants and animals to improve flavor in the starting materials of food products.

To illustrate some trends and developments in flavor chemistry research, chapters have been included on the importance of enantiomers and the biogenesis of some chiral compounds, production and mechanisms of natural and chemically formed flavor compounds, and a few recent examples of chemical investigations of characteristic flavors.

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Chapter 1

New Trends and Developments in Flavor Chemistry

An Overview

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This chapter gives an overview of new trends and developments in flavor chemistry. One important development was made possible by advances in analytical methodology, that is, the identification of numerous compounds with known flavor characteristics. As more and more compounds are correlated with characteristic flavors, there is a trend to study flavor precursors and to explain how flavor is developed and released. Many of the newest developments in flavor chemistry are in the area of flavor production from plant and animal sources; this trend has come about because of the public's fear of the words "chemical" and "synthetic". In this chapter, words such as these are discussed in terms of the public's perception of them versus a chemist's viewpoint. Another new trend is to understand the chemical reactions involved in the processing and storage of foods in order to bring foods to consumers at optimum acceptability.

Prior to the 1950's only about 500 flavor compounds were known (1). Since then, with the advent of modern instrumentation, thousands of compounds have been characterized in hundreds of different foods (2). There have been many books published on flavor research workshops and symposia, some of which are held on a periodic basis and some on special occasions and topics, covering various aspects of flavor (3-20). Also, there are many excellent reviews which every serious flavor chemist should consult (21-43).

Advances in analytical methodology introduced in the 1960's were applied from the early 1970's (3, 5, 12, 13, 14). Previous to gas chromatography, fractional distillations and column chromatography of colored derivatives were the primary means of separations. Size of sample required for distillation is, of course, enormous compared to what is required for gas chromatography. The resolution of separation by gas chromatography is far superior to that attained by

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fractional distillation. Also, the advent of infrared, nuclear magnetic resonance, and mass spectrometry has made it possible to make structural determinations with micro amounts. Thus, with the use of modern analytical methods, the number of compounds with known flavor characteristics increased in the 1970's and 1980's. These advances have set the stage for the present trends and developments in flavor chemistry.

Many of the newest developments in flavor chemistry are in the area of flavor production from plant and animal sources; hence, signifying the popularity of the term "biotechnology". This trend has come about because of the fear of the public of the words "chemical" and "synthetic".

The word "natural" is used in opposition to the word "synthetic" with the connotation that "natural" products are safer than "synthetics", but there are many toxins made by plants and animals which are very detrimental to man (44-47). Moreover, whether chemicals are made in flasks by man or made by plants and animals, no compounds are made on earth other than those permitted by the laws of nature. Therefore, all molecules on this earth are "natural". This definition is from a chemist's viewpoint, and is in agreement with a dictionary definition of "characteristic of or explainable by the operations of the physical world".

However, The Food and Drug Administration definitions (48) are: "The term 'artificial flavor' or 'artificial flavoring' means any substance, the function of which is to impart flavor, which is not derived from a spice, fruit or fruit juice, vegetable or vegetable juice, ... , or fermentation products thereof. ... The term 'natural flavor' or 'natural flavoring' means the essential oil, oleoresin, essence or extractive, protein hydrolysate, distillate or any product of roasting, heating or enzymolysis, which contains the flavoring constituents derived from a spice, fruit or fruit juice, vegetable or vegetable juice, edible yeast, herb, bark, bud, root, leaf or similar plant material, meat, seafood, poultry, eggs, dairy products, or fermentation products thereof." It is this set of words, "or fermentation products thereof", which has set off a flurry of activity in biotechnology in order to use the words "natural flavoring" on the label of food products.

The public has an unbased fear of "chemicals" and "synthetics" and an unbased confidence in "natural" compounds and products. The public should be educated that there are no differences in the molecules used in flavorings which are made by man in flasks or by plants and animals. However, because of this fear, there is a trend in the use of "naturals", materials from plants and animals (including microorganisms) obtained by biotechnology, over "synthetics", materials from chemical laboratories.

Plants and animals have been selected by classical genetic methods for optimum yield, color, texture, disease resistance, etc. It is time for plants and animals to be selected for optimum flavor. In

this long range plan, flavor chemistry will help in the selection of plants and animals for acceptability whether it is by the classical or modern genetic engineering methods.

In the short range plan, raw materials can be harvested, processed, stored and shipped to bring more flavorful food products to consumers. Very few food products are used directly as grown on the farm. Grains must be milled, made into flour, and then baked to make breads and other cereal products. Fruits and vegetables must be picked at a time to give optimum flavor and texture. Most meats, red or white flesh, are almost flavorless until heated. Chemical reactions involved in the above situations must be understood in order to bring foods to consumers at optimum acceptability.

The development of modern analytical methods has permitted the examination of volatiles from fresh fruit to determine when to pick the fruit. It is the usual concept that fruits are at their best when picked "tree-ripe". However, in extreme cases, as with bananas and pears, these fruits must be picked when hard and green and be permitted to soften and ripen off the tree. If these fruits are permitted to ripen on the tree, they become mealy and unacceptable. Some fruit, like strawberries and peaches, are of best quality when left to ripen on the plants. Apples have been shown to have the best aroma if picked almost ripe and develop the most aroma about a week or two after picking.

Man has used biotechnology for converting raw materials to food products for many centuries. Production of beer is thought to date back to about 6000 BC in ancient Babylonia. The predecessors of soy sauce and miso seem to have originated in China some 2500 years ago. Man has been using products altered by microorganisms, and has learned by trial and error which of the products are safe to eat and which are not. Modern scientific methods are now being applied to explain what chemical and physical alterations are accomplished by microorganisms. Also, further advancements in biotechnology will probably be made to make such systems even more efficient, perhaps even bypass living organisms by using only enzymes. As more and more compounds are correlated with characteristic flavors, there is a trend to study flavor precursors and to explain how flavor is developed and released, especially since now it can be determined exactly which enantiomer is making the contribution to a characteristic aroma.

The era of publishing a large number of compounds identified as to chemical structures is slowly changing to an era in which constituents are identified as to which are the important contributors to the characteristic odors. More and more sensory analyses are stating odor threshold values as well as odor quality.

In the evaluation of contribution to taste, amino acids and peptides are being studied as to sweet, salty, bitter, sour and umami [brothy mouth-feel, see (19)] sensations. In the production of gravies and soups, proteins are hydrolyzed to smaller molecules which evoke

more taste sensations than do the large protein molecules. Systematic studies of amino acids and peptides are providing interesting data which will be useful in optimizing conditions to yield the greatest amount of acceptable tastes and minimizing undesirable tastes.

As analytical methodology is improved, the known number of compounds contributing to flavor will be increased, and flavor chemistry will become more applied. Industrial organizations will be able to utilize the information gained in fundamental research to improve the quality of their products. At the same time, there will be more of a data base on which to build a better understanding of the mechanisms of perception of taste and olfaction.

As the demand for natural flavors increases, and as constituents contributing to such flavors are identified, flavor chemistry will be applied in the biotechnological production of such flavors. Also, processing methods will be followed to retain most of the fresh flavors of raw materials. Cases in which flavor is developed during processing, modern analytical methods will be applied to adjust processing conditions to produce the optimum desirable flavors. Thus, flavor chemistry has reached a stage where it is now being applied to improve the flavor of foods, fresh and processed, reaching many consumers.

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Chapter 2

Biosynthesis of Chiral Flavor and Aroma Compounds in Plants and Microorganisms

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Capillary gas chromatographic determination of optical purities, investigation of the conversion of potential precursors, and characterization of enzymes catalyzing these reactions were applied to study the biogenesis of chiral volatiles in plants and microorganisms. Major pineapple constituents are present as mixtures of enantiomers. Reductions, chain elongation, and hydration were shown to be involved in the biosynthesis of hydroxy acid esters and lactones. Reduction of methyl ketones and subsequent enantioselective metabolism by *Penicillium citrinum* were studied as model reactions to rationalize ratios of enantiomers of secondary alcohols in natural systems. The formation of optically pure enantiomers of aliphatic secondary alcohols and hydroxy acid esters using oxidoreductases from baker's yeast was demonstrated.

The world-wide trend to "natural" flavor and aroma has significantly increased interest in biogenetical pathways leading to volatiles in natural systems. For chiral compounds the exploration of potential biosynthetic routes is even more important, because chemical syntheses are often difficult and expensive; in many cases however sensory qualities of enantiomers are different (1-3). In our current studies of chiral volatiles in plant and microbial systems we use different analytical approaches. (a) Capillary gas chromatographic separations of diastereoisomeric derivatives are used to determine the configurations of chiral constituents at trace levels. (b) Chemically synthesized (labeled) precursors are added to fruit tissues and microorganisms. Their transformation into chiral constituents is investigated by means of capillary gas chromatography/mass spectrometry; the stereochemical course of these metabolizations is followed. (c) Enzymes catalyzing the stereospecific conversion of precursors to chiral compounds are isolated and characterized; commercially available enzymes are investigated as model systems to elucidate the stereochemical course of biogenetical pathways. The combination of these methods revealed some new aspects of the biosynthesis of chiral compounds in natural systems.

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Naturally Occurring Configurations of Pineapple Volatiles

A distinct feature of the spectrum of volatiles isolated from pineapple (*Ananas comosus* (L.) Merr.) is the presence of numerous chiral components: 3- and 5-hydroxy esters, 3-, 4- and 5-acetoxy esters, and γ - and δ -lactones are prominent pineapple flavor and aroma constituents (4-7). Capillary gas chromatographic separation of diastereoisomeric derivatives of (S)-(+)- α -methoxy- α -trifluoromethylphenylacetic acid chloride (MTPA) and (R)-(+)-phenylethylisocyanate (PEIC) revealed that these chiral pineapple components are not contained in optically pure form, but as mixtures of enantiomers. The enantiomeric ratios listed in Table I confirmed findings of previous investigations of pineapples (8). Ratios of enantiomers rather than optically pure constituents have also been observed in other fruits, such as passion fruit and mango (9).

Table I. Concentrations and enantiomeric compositions of chiral constituents in firm-mature and soft-ripe pineapples

compound	concentration (ppb)		increase (%) ^a	enantiomeric composition ^b			
	firm-mature	soft-ripe		firm-mature		soft-ripe	
				% (S)	% (R)	% (S)	% (R)
methyl 3-hydroxy-hexanoate	310	390	26	84	16	86	14
methyl 3-acetoxy-butanoate	120	380	217	- ^c		83	17
methyl 3-acetoxy-hexanoate	1630	3680	126	91	9	93	7
methyl 4-acetoxy-hexanoate	480	540	13	73	27	75	25
methyl 5-acetoxy-hexanoate	1200	1610	34	64	36	62	38
methyl 4-acetoxy-octanoate	-	<30	-	-		- ^c	
methyl 5-acetoxy-octanoate	1000	1050	5	- ^b		51	49
γ -hexalactone	630	1360	116	76	24	80	20
δ -hexalactone	310	320	3	16	84	37	63
δ -octalactone	750	580	-	49	51	53	47

a) ripening over 5 days at room temperature;

b) determined by GC-investigation of MTPA- and PEIC-derivatives, respectively;

c) enantiomeric composition not determined.

The biogenesis of volatiles in plant systems is a very dynamic process. Changes in the activities of enzymes and pathways at different stages of maturity and ripeness might influence not only the concentrations but also the optical purities of chiral constituents. Therefore we subjected "firm-mature" pineapples to post-harvest ripening at room temperature over a period of 5 days. The concentrations of all chiral components (except δ -octalactone) increased significantly. The enantiomeric compositions, however, remained nearly unchanged during this ripening process (Table I). This constancy of enantiomeric ratios at different physiological stages of the fruits is a very important result. The independence of optical purities on maturity and ripeness is one of the premises to use the investigation of chiral constituents for detection of adulterations in fruit products (10).

Addition of Oxocompounds to Pineapple Tissue

Mixtures of enantiomers rather than optically pure compounds can be rationalized by different assumptions: (a) one enzyme with low enantioselectivity catalyzes the biogenetical process, (b) at least two enzymes with different enantioselectivities compete in the reaction, and (c) different pathways eventually leading to the same final products, however with opposite configurations, are involved. By addition of chemically synthesized precursors we aimed to trace some of the biogenetical routes involved in the biogenesis of chiral pineapple volatiles.

The distribution of metabolites obtained after incubation of pineapple slices with keto acids and keto esters, potential precursors of the corresponding hydroxy compounds, is summarized in Table II. The metabolization steps comprise esterification, reduction to hydroxy compounds, formation of acetoxy esters, and cyclization to the corresponding lactones. Metabolization rate and distribution of formed products strongly depend on the structures of the precursors. The detection of these metabolites proves the enzymatic capability of pineapple tissue to catalyze these conversions, an aspect which might be interesting for future use of pineapple tissue cultures in the production of chiral compounds.

Capillary gas chromatographic investigation of diastereoisomeric derivatives revealed that in analogy to results obtained without precursors the chiral metabolites are present as mixtures of enantiomers. However for only a few of these compounds the ratios of enantiomers are identical with those determined in pineapple without precursors. The enantiomeric compositions of ethyl 3-hydroxyhexanoate and ethyl 3-acetoxyhexanoate are almost opposite to those determined for the naturally occurring methyl esters. δ -Octalactone obtained after addition of 5-oxooctanoic acid to pineapple tissue is almost optically pure (92% S); on the other hand δ -octalactone is naturally present in pineapple tissue as nearly racemic mixture (Table I,8).

One might conclude from these differences in the optical purities that the reduction of ketoprecursors can not be the major pathway active in the biosynthesis of hydroxy esters, acetoxy esters, and lactones in pineapple. However, when interpreting these results it must be considered that in the case of a competition of two en-

Table II. Concentrations and enantiomeric compositions of metabolites formed after incubation of pineapple slices

<u>precursor products</u>	concentration (ppb)		degree of metabo- lization (%)	enantiomeric composition ^b	
	before incubation ^a	after incubation ^a		% (S)	% (R)
<u>ethyl 3-oxohexanoate</u>			85		
ethyl 3-hydroxyhexanoate	-	34540		18	82
ethyl 3-acetoxyhexanoate	-	5850		31	69
<u>ethyl 4-oxohexanoate</u>			12		
ethyl 4-hydroxyhexanoate	-	3200		11	89
ethyl 4-acetoxyhexanoate	-	180		- ^c	
γ -hexalactone	300	2380		23	77 ^d
<u>ethyl 5-oxohexanoate</u>			23		
ethyl 5-hydroxyhexanoate	-	7250		67	33
ethyl 5-acetoxyhexanoate	-	1950		76	24
δ -hexalactone	40	3050		60	40 ^d
<u>methyl 5-oxohexanoate</u>			9		
methyl 5-hydroxyhexanoate	<20	4670		50	50
methyl 5-acetoxyhexanoate	430	1750		85	15
δ -hexalactone	50	1090		44	56
<u>5-oxohexanoic acid</u>			2.5		
methyl 5-oxohexanoate	-	2440		-	
methyl 5-hydroxyhexanoate	<20	1960		27	73
methyl 5-acetoxyhexanoate	319	457		- ^c	
δ -hexalactone	56	1643		40	60 ^d
<u>5-oxooctanoic acid</u>			3		
methyl 5-oxooctanoate	-	500		-	
methyl 5-hydroxyoctanoate	<10	540		68	37
methyl 5-acetoxyoctanoate	150	160		- ^c	
δ -octalactone	520	6920		8	92 ^d
<u>5-oxodecanoic acid</u>			4		
methyl 5-oxodecanoate	-	140		-	
methyl 5-hydroxydecanoate	-	-		-	
methyl 5-acetoxydecanoate	-	-		-	
δ -decalactone	-	13800		20	80 ^d

a) incubation time: 20 hours; concentration of precursors: oxoesters: 0.4 mmol/kg; oxoacids: 2.0 mmol/kg;

b) determined by GC-investigation of MTPA- and PEIC-derivatives, respectively;

c) enantiomeric composition not determined;

d) enantiomeric composition determined by GC-investigation of PEIC-derivatives of the corresponding diols.

zymes, showing different enantioselectivities and different K_m -values for a common substrate, the concentration of this substrate decisively determines the reaction rates of the enzymes and thus the enantiomeric composition of the product. Therefore the "unnaturally" high concentration of a precursor, as a result of the addition of a relatively high amount of the chemically synthesized compound to the plant tissue, may be the reason that optical purities of products are different from those without precursors.

The presence of at least two enzymes with different enantioselectivities depending on the structures of the substrates, comparable to the oxoacid ester reductases in baker's yeast (11), is indicated by the different optical purities obtained after incubation of 5-oxohexanoic acid, methyl 5-oxohexanoate, and ethyl 5-oxohexanoate (Table II). Variations of the concentrations of added precursors also resulted in different optical purities of metabolites (12).

Addition of Labeled Precursors to Pineapple Tissue

If unlabeled precursors are added the investigation of pathways is mainly based on quantitative data and requires significant increases of metabolites. The use of deuterium labeled compounds offers the possibility to follow conversions of precursors with lower metabolization rates, because the formed products can be detected based on their content of deuterium by using the more sensitive method of capillary gas chromatography-mass spectrometry. This technique revealed that two independent pathways starting from different precursors contribute to the formation of δ -octalactone in pineapple.

Figure 1 shows part of a reconstructed ion chromatogram of a pineapple aroma extract isolated after incubation of pineapple slices with 3-hydroxyhexanoic acid-3- d_1 . GC-MS detection of deuterated compounds showed that the following pathways are active: (a) esterification leading to methyl and ethyl esters, (b) dehydration to (E)-2- and (E)-3-hexenoates, and (c) chain elongation to methyl 5-hydroxy octanoate followed by acetylation (methyl 5-acetoxyoctanoate) and cyclization (δ -octalactone).

On the other hand deuterated δ -octalactone was also detected after addition of (Z)-4-octenoic acid-2,2- d_2 to pineapple tissue (Figure 2). Hydration of the double bond followed by cyclization of the intermediates 4-hydroxyoctanoic acid-2,2- d_2 and 5-hydroxyoctanoic acid-2,2- d_2 leads to γ -octalactone-2,2- d_2 and δ -octalactone-2,2- d_2 , respectively.

The formation of δ -octalactone by three independent pathways (a) reduction of 5-oxoprecursors, (b) chain elongation of 3-hydroxyhexanoate, and (c) hydration of (Z)-4-octenoic acid demonstrates the complexity of the biosynthesis of chiral compounds in natural systems.

Oxidoreductases Catalyzing the Enantioselective Reduction of Oxoacid Esters in Baker's Yeast

Early investigations of reactions catalyzed by Saccharomyces cerevisiae revealed that the stereochemical course of the reduction of 3-oxoacids is influenced by the chain lengths of the substrates:

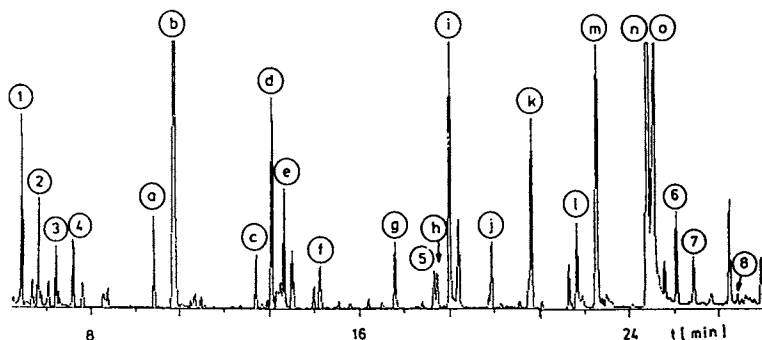


Figure 1. Part of a capillary gas chromatogram (reconstructed ion detection) of an aroma extract of pineapple tissue, isolated after addition of 3-hydroxyhexanoic acid-3-d₁ (CP Wax 52 CB column, 50 m x 0.32 mm i.d., df = 0.25 μm; temperature program 70-220 °C; 4 °C/min).

- | | |
|--|-----------------------------------|
| (1) ethyl hexanoate-3-d ₁ | (d) methyl 3-methylthiopropionate |
| (2) methyl (Z)-3-hexenoate-3-d ₁ | (e) methyl 3-acetoxybutanoate |
| (3) methyl (Z)-2-hexenoate-3-d ₁ | (f) O-methyl furaneol |
| (4) ethyl (E)-3-hexenoate-3-d ₁ | (g) methyl 3-hydroxyhexanoate |
| (5) ethyl 3-hydroxyhexanoate-3-d ₁ | (h) γ-hexalactone |
| (6) methyl 5-acetoxyoctanoate-5-d ₁ | (i) methyl 3-acetoxyhexanoate |
| (7) δ-octalactone-5-d ₁ | (j) 3-methylthiopropanol-1 |
| (8) methyl 5-hydroxyoctanoate-5-d ₁ | (k) methyl 4-acetoxyhexanoate |
| (a) methyl octanoate | (l) δ-hexalactone |
| (b) octanol-2 (internal standard) | (m) methyl 5-acetoxyoctanoate |
| (c) dimethyl malonate | (n) β-phenylethanol |
| | (o) γ-octalactone |

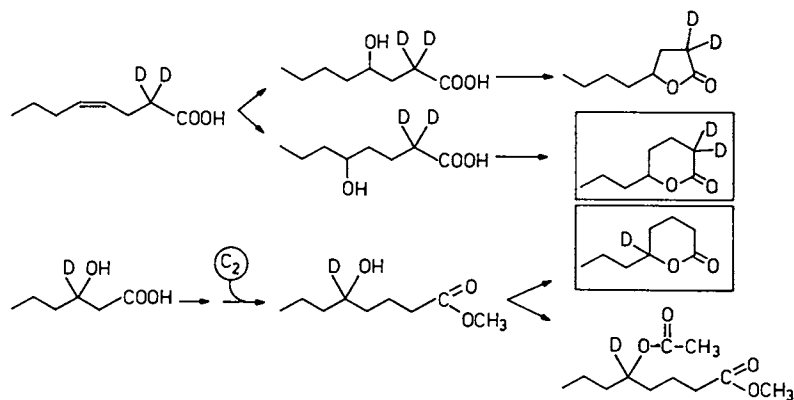


Figure 2. Pathways active in the biosynthesis of δ-octalactone in pineapple.

3-oxobutanoic acid is reduced to (S)-3-hydroxybutanoic acid (13), whereas the reduction of 3-oxohexanoic acid leads to the (R)-configured hydroxyacid (14). Sih et al. (15) demonstrated that the optical purities of 3-hydroxyacid esters obtained by yeast catalyzed reduction can be controlled by variation of the structures of the educts (e.g. by different chain lengths of acid and alcohol moieties, respectively, of the 3-oxoacid esters). They postulated the existence of two enzymes with different enantioselectivities and different substrate specificities, the so-called (R)- and (S)-enzymes, which are competing for the substrate.

By means of streptomycin sulfate treatment, Sephadex G-25 filtration, DEAE-Sephadex CL-6B chromatography, Sephadex G-150 filtration, and hydroxyapatite chromatography we succeeded in isolating and purifying two NADPH-dependent oxidoreductases from enzyme extracts of *Saccharomyces cerevisiae*, which catalyze the enantioselective reduction of 3-oxoacid esters to (S)- and (R)-3-hydroxyacid esters (11). The elution pattern of enzymes reducing ethyl 3-oxohexanoate obtained after DEAE-Sephadex-CL-6B chromatography is shown in Figure 3.

The (S)-enzyme (relative molecular mass 48000 -50000) reduced 3-oxoacid esters, 4-oxo and 5-oxoacids and esters enantioselectively to (S)-hydroxy compounds. The K_m -values for ethyl 3-oxobutanoate, ethyl 3-oxohexanoate, 4-oxopentanoic acid and 5-oxohexanoic acid were determined as 0.9 mM, 5.3 mM, 17.1 mM and 13.1 mM, respectively.

The (R)-enzyme (molecular mass 800000) was shown to be identical with a subunit of the fatty acid synthase complex. It reduced 3-oxoacid esters specifically to (R)-hydroxyacid esters. K_m -values for ethyl 3-oxobutanoate and ethyl 3-oxohexanoate were determined as 17.0 mM and 2.0 mM, respectively. Intact fatty acid synthase showed no activity in catalyzing the reduction of 3-oxoacid esters.

Opposite enantiomers of 3-hydroxyacid esters of different chain lengths in fruits, such as yellow passion fruit (16) and the influence of the structures of oxoprecursors on the optical purities of 3-hydroxyacid derivatives in incubation experiments with pineapple indicate a competition of oxidoreductases in plant systems comparable to baker's yeast.

Secondary Alcohols in Plants

Capillary gas chromatographic investigation of diastereoisomeric derivatives revealed that in some fruits, such as passion fruits (9) and blackberries (17), secondary alcohols and their esters are contained in almost optically pure form. On the other hand corn (*Zea mays*) contains aliphatic secondary alcohols as mixtures of enantiomers; the ratios depend upon the chain lengths of the alcohols. Heptan-2-ol is present mainly as (R)-enantiomer; with increasing chain length the proportion of (S)-enantiomer increases. A similar distribution has been determined in coconut (Figure 4).

To elucidate these results we decided to study the stereochemical course of the formation of secondary alcohols by microorganisms and purified enzymes as model systems.

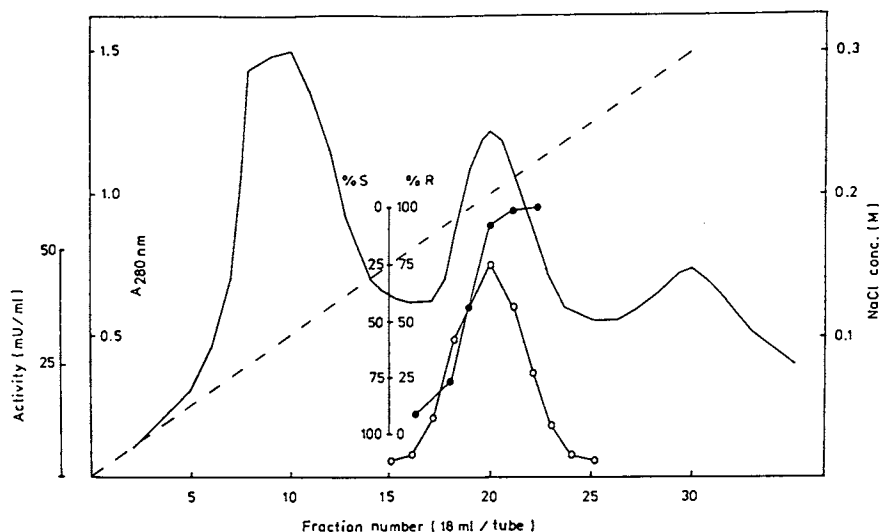


Figure 3. Elution pattern of enzymes reducing ethyl 3-oxohexanoate on DEAE-Sepharose CL-6B chromatography with increasing NaCl concentration. (O) enzyme activity; assay: 1.5 ml 200 mM Tris-HCl buffer (pH 7.2), 0.2 ml 1 mM NADPH solution, 0.1 ml 50 mM ethyl 3-oxohexanoate (emulsified in an aqueous solution of 5 % propyleneglycol) and 0.05 ml enzyme solution; measuring the absorbance at 340 nm at 25 °C. (●) enantiomeric composition (GC separation of MTPA-derivatives) of the formed ethyl 3-hydroxyhexanoate; assay: active fractions (tubes 16 to 23), ethyl 3-oxohexanoate (2 mg) and NADPH (1.5 mg) in a total volume of 3 ml 0.1 Tris-HCl buffer, pH 7.2, for 6 hours. (—) absorbance 280 nm. (---) concentration of NaCl. (Reproduced with permission from Ref.11. Copyright 1988 FEBS.)

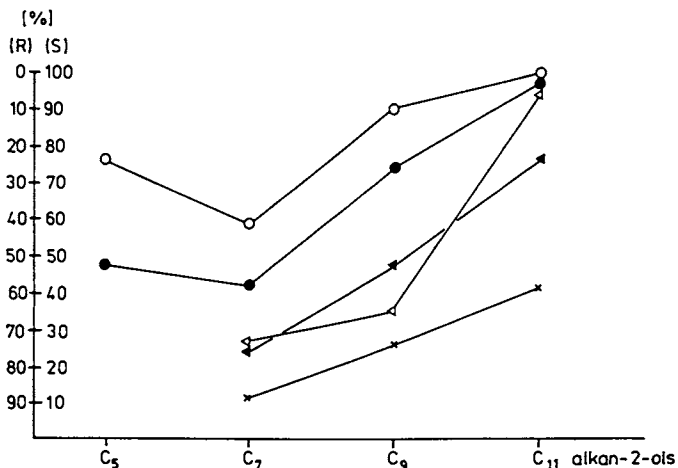


Figure 4. Enantiomeric compositions of secondary alcohols isolated from corn (x = Golden Jubilee, Δ = Golden Bantam, ▲ = Bonanza) and coconut (●) and obtained after reduction of methyl ketones in a shake culture of *Penicillium citrinum* after 68 h (○).

Stereochemical Course of the Formation of Secondary Alcohols by *Penicillium citrinum*

The formation of methyl ketones can be rationalized by a modified β -oxidation of fatty acid precursors (18). This process, caused by some moulds, mainly deteriorates short chain fatty acids and leads to the so-called "ketonic-rancidity". Kinderlerer et al. (19-21) demonstrated the formation of methyl ketones and secondary alcohols by some xerophilic fungi isolated from coconuts. After deacylation and decarboxylation of the β -ketoacyl-CoA-intermediates the last step of the biogenetical sequence is the reduction of the corresponding methyl ketones.

To study the stereochemical course of this reduction we added a homologous series of aliphatic methyl ketones (C₇-C₁₁) as precursors to a shake culture of *Penicillium citrinum*. Results obtained for heptan-2-one and nonan-2-one are summarized in Table III. The degree of reduction depends on the chain lengths; maximum conversion was observed for nonan-2-one. *Penicillium citrinum* preferentially catalyzes the formation of (S)-alkan-2-ols. However the optical purities of the formed alcohols were also influenced by the chain lengths. The highest optical purity was obtained for decan-2-ol (96 % enantiomeric excess, e.e. (S)). With decreasing chain length the proportion of (R)-alcohol increased; heptan-2-ol was isolated with a maximum purity of only 64% (S):36% (R). These results indicate the presence of at least two chain length specific oxidoreductases with different enantioselectivities comparable to the oxoester reductases in baker's yeast.

Table III: Quantitative distribution and enantiomeric compositions of secondary alcohols obtained by reduction of methyl ketones with *Penicillium citrinum*

reaction-time (h)	heptan-2-ol		nonan-2-ol	
	concentration (ppm)	enantiomeric composition ^a %(S) %(R)	concentration (ppm)	enantiomeric composition ^a %(S) %(R)
4	1.1	58 42	16.2	96 4
7	2.5	59 41	22.5	97 3
11	4.9	60 40	24.5	97 3
21	6.7	63 37	23.0	97 3
32	6.9	64 36	20.2	96 4
46	6.7	63 37	14.2	94 6
69	5.5	60 40	4.5	88 12
96	2.2	42 58	0.1	56 44

a) determined by GC investigation of MTPA- and PEIC-derivatives

There is however a second phenomenon decisively influencing the optical purities of the alcohols formed in the course of *Penicillium citrinum* catalyzed reduction. The formed alcohols are metabolized again; this metabolization proceeds enantioselectively. The preferentially formed (S)-enantiomer is preferentially metabolized. As shown in Table III the optical purity (% enantiomeric excess, e.e.) of nonan-2-ol decreases from 92% e.e. (S) to 12% e.e. (S). Heptan-2-ol is finally present mainly as (R)-enantiomer. The metabolization steps are currently under investigation; one of the pathways is a hydroxylation leading to hydroxy ketones and diols. Figure 5 presents structures of hydroxylated metabolites obtained from nonan-2-one.

Quantitative distribution and optical purities of the secondary alcohols obtained after fermentation with *Penicillium citrinum* are very similar to those isolated from coconut or corn (Figure 4). A combination of stereospecific reduction and following enantioselective metabolization may be one of the keys to explain the ratios of enantiomers of aliphatic secondary alcohols observed in natural systems.

Formation of Optically Pure Secondary Alcohols by Yeast Alcohol Dehydrogenase

Alcohol dehydrogenase (ADH) from baker's yeast (*Saccharomyces cerevisiae*) is a major enzyme involved in the oxidation of secondary alcohols and the reduction of methyl ketones, respectively. The stereochemical course of the oxidation has been investigated using racemic butan-2-ol and octan-2-ol as substrates; only the (S)-enantiomers of these alcohols were converted to the corresponding ketones (22,23). On the other hand the enantioselectivity of yeast ADH in the reduction of ketones has been unclear. When whole yeast cells are employed the reduction of methyl ketones also leads to mainly (S)-configured alcohols, however the optical purities of these alcohols are only moderate (24). MacLeod et al. (24) rationalized this lack of 100% stereoselectivity by the assumption that alcohol dehydrogenase may be the only enzyme involved in this reduction but that it is only partially stereoselective when acting on these unnatural substrates.

By means of capillary gas chromatographic determination of the optical purities of formed products we could demonstrate that yeast alcohol dehydrogenase catalyzes not only the oxidation of racemic secondary alcohols but also the reduction of the corresponding methyl ketones in highly stereoselective manner.

Starting from a racemic mixture of an alkan-2-ol only the (S)-enantiomer is converted to the corresponding ketone. A complete oxidation, necessary to eventually obtain the remaining alcohol in optically pure form, was achieved by regeneration of NAD in a coupled reduction of decanal to decanol. Aliphatic methyl ketones were reduced stereoselectively to (S)-alkan-2-ols. NADH, required for this process, was generated from NAD in a coupled oxidation of ethanol to acetaldehyde (Figure 6). The capillary gas chromatographic separations of diastereoisomeric derivatives of a series of aliphatic secondary alcohols obtained by these yeast ADH catalyzed reactions are shown in Figure 7.

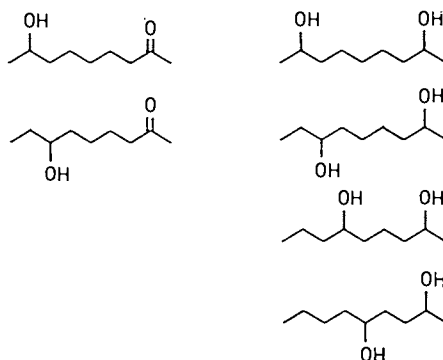


Figure 5. Hydroxylated metabolites isolated after addition of nonan-2-one to a shake culture of *Penicillium citrinum*.

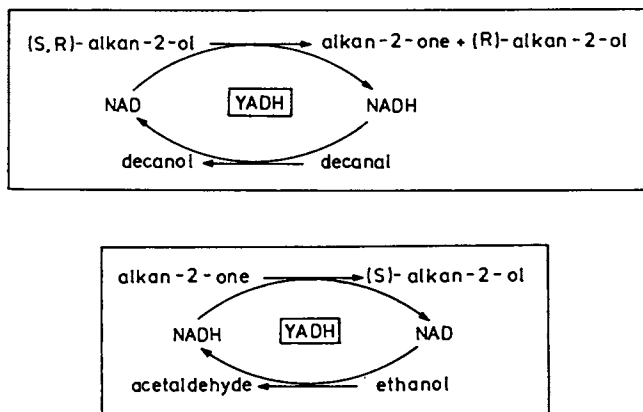


Figure 6. Formation of optically pure secondary alcohols by yeast alcohol dehydrogenase catalyzed oxidation of racemic mixtures of alkan-2-ols and reduction of alkan-2-ones.

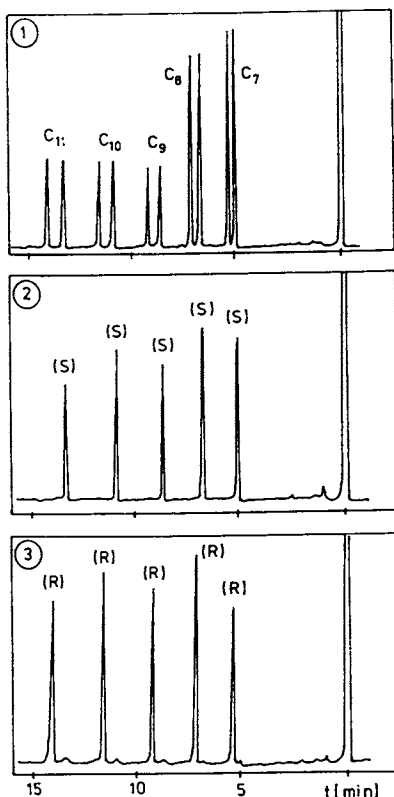


Figure 7. Capillary GC-separation of (R)-(+)-PEIC derivatives of C₇-C₁₁-alkan-2-ols (DB 210, 30 m x 0.32 mm i.d., df = 0.25 μm; 150-240 °C; 2 °C/ min.).

(1) Racemic alcohols;

(2) (S)-enantiomers obtained by reduction of about 2 mg of alkan-2-ones in 4 ml Tris-HCl buffer pH 8.1 (0.1 M) containing 0.2 ml ethanol, 3 mg NAD and 2 mg YADH (specific activity towards ethanol: 280 U/ mg); incubation time: 6 hours;

(3) (R)-enantiomers obtained by oxidation of about 1 mg of racemic alkan-2-ols in 4 ml buffer pH 8.1 containing 4 mg decanal, 3 mg NAD and 2 mg YADH; incubation time: 6 hours.

These results demonstrate that whole cells of Saccharomyces cerevisiae must contain oxidoreductases others than alcohol dehydrogenase which are involved in the reduction of methyl ketones and catalyze the formation of (R)-alkan-2-ols.

Outlook

The examples presented in this chapter demonstrate that a combination of various analytical approaches and the selection of suitable model systems can add valuable information to our knowledge about pathways and enzymes involved in the biosynthesis of chiral volatiles. Some of the techniques need further improvement, e.g. by use of radioactively labeled precursors the detection threshold of metabolites can be lowered significantly; addition of precursors in concentrations comparable to those in natural plant or microbial systems would be possible. The investigation of the enantioselectivity of enzymes has to be emphasized, eventually not only enzymes commercially available or easily accessible in microorganisms but also those active in plant systems have to be studied.

The knowledge about the stereochemical properties of enzymes catalyzing the biosynthesis of chiral volatiles is not only interesting from a strictly scientific standpoint of view; it is also an essential basis for future improvement of natural flavor and aroma by genetical engineering of plants and microorganisms.

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Chapter 3

Aroma Development in Ripening Fruits

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Instrumental aroma analysis, involving headspace sampling, analysis and identification of aroma compounds by gas chromatography-mass spectrometry allows evaluation of the different flavor determining parameters in fruits. In apples e.g. aroma compounds, mainly esters, are formed gradually during ripening in a manner which parallels the respiration climacteric towards a maximum. Headspace gas chromatography permits following the complete ripening process. Measurement during the early picking period, allows prediction of the earliest acceptable harvest date for storage apples. After storage in controlled atmosphere (CA-storage) the normal ripening pattern is disturbed and ester production diminishes as a function of storage time. Volatile analysis by non-destructive headspace techniques is also an interesting tool for flavor formation studies e.g. by treatment of intact apples with ester precursors (carboxylic acids, aldehydes, alcohols).

It is generally recognised that in fruit and vegetable production more attention should be given to the hidden sensory quality parameters, such as flavor and texture. These quality attributes are the result of a number of pre- and post-harvest factors and are closely related with fruit ripening. Paillard distinguishes external and internal factors influencing aroma formation in fruits (1). The first ones are associated with the culture of the plant and post harvest treatments, the second are in connection with the metabolic regulation of the fruit. A survey is given in Figure 1.

It is not possible to cover these different aspects for a variety of fruit products. Therefore we selected to discuss the knowledge of the influencing parameters on apple flavor and to focus on some recent analytical achievements, which are of importance for the study of these effects. Excellent work has already been done concerning the separation and identification of aroma giving compounds. The state of the art up to 1982 on apple flavor has been clearly reviewed by Dimick and Hoskin (2), covering important studies by

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EXTERNAL FACTORS

PRE-HARVEST : SOIL/HYDROPONIC CULTURE
 FERTILIZATION
 CLIMATE/IRRIGATION

PICKING DATE-MATURITY

POST-HARVEST TECHNOLOGY : DURATION OF STORAGE
 STORAGE CONDITIONS :
 TEMPERATURE
 HUMIDITY
 GAS COMPOSITION

INTERNAL FACTORS

GENETIC CONTROL : CULTIVAR
 METABOLIC REGULATION : ETHYLENE
 RESPIRATION

Figure 1 . Factors influencing flavor formation in fruits.

Drawert (3), Flath (4), Guadagni (5), Williams (6) and many others. Some compounds have been claimed to be important contributors to apple aroma : e.g. ethyl 2-methylbutanoate, n.hexanal, trans-2-hexenal and 4-methoxyallylbenzene. However it seems that apple aroma is not a matter of a limited number of character impact compounds but is due to complex mixture of alcohols, aldehydes, C₁-C₆ esterified acids, estragol and terpenes (7). Recently Yajima et al. (8) identified 22 new apple flavor components in the steam distillate of Kogyoku apples, which correspond to American Jonathan apples. In an interesting approach using "Charm" analysis on forty different apple cultivars Cunningham et al. indicated the importance to apple odour of β -damascenone, butyl-,3-methylbutyl- and hexyl hexanoates, along with ethyl, propyl and hexyl butanoates.

Flavor formation in fruit products has also extensively been reviewed (10). A distinction can be made between the primary aroma components, which are biosynthesized by the whole fruit and secondary aroma compounds (e.g. hexanal, 2-hexenal), formed after disruption of the cells during processing or chewing (11). The importance of the peel for aroma formation has also been stressed by several authors (12). An extensive literature on the respiration climacteric (13), the role of ethylene (14) and the enzymes and substrates required for biosynthesis is available (15).

The topic of apple flavor should also be considered in relation to the modern developments in isolation and preparation technology. The important influence of the sample preparation techniques on the composition of biologically derived aromas was recently reviewed by Parliament (16).

The goal of this article is to present a procedure for aroma analysis, consisting of dynamic headspace sampling of intact fruits, followed by high resolution gas chromatography - mass spectrometry. This procedure allows evaluation of the influence of cultivar, picking date and controlled atmosphere storage on aroma development in apples. The analytical system is also of importance for studying biogenesis of aroma components in intact fruits.

Analysis Technology: Dynamic Headspace Gas Chromatography

Dynamic headspace sampling on Tenax GC, by trapping volatiles, liberated during disintegration of fruits, has been previously described by the authors (17,18). This procedure allows the isolation of an instant aroma and imitates the eating mechanism. Dynamic headspace sampling also offers the possibility for isolation of volatiles, released from intact apples, which in the case of apple aroma, allows following the complete ripening process. A typical apparatus for isolation of volatiles from intact apples is presented in Figure 2. The dessicators are continuously flushed with air and at regular intervals for sampling a Tenax adsorber is attached to the outlet of the dessicator. For quantitative adsorption high amounts of Tenax GC are used: 5 g of Tenax GC 60/80 mesh, packed in glass tubes (i.d. 1.6 cm, length 10 cm). Sample recovery can be performed by heat desorption and collection in a cold trap, which allows a sharp injection on 0.5 mm i.d. capillary columns. Therefore the gas chromatographs are modified with a selfconstructed injection system, consisting of a desorption oven and a thermostated two-position six-port, high temperature injection valve. The full experimental details of the analysis system have been described before (19). As an illustration of the results obtained by this system in Figure 3 the chromatogram of Spartan variety apple volatiles, isolated by dynamic headspace sampling of the intact fruits, is presented together with the identified compounds.

Influence of Picking Date

As aroma is one of the key factors in flavor quality, it can be used as a criterion for evaluation of flavor quality of apples. By following the evolution of the volatile composition a complete picture of the dynamic flavor quality process can be obtained. In Figure 4 the sum of esters is presented as a function of days of ripening in standard conditions (18°C) for Golden Delicious apples.

Late picked apples show an immediate aroma development and reach a considerable higher maximum compared to early picked apples. From this picture one can conclude that for optimum apple quality the picking date should be related to the consumption period.

Figure 4 also illustrates the well known fact that too early picked apples will never develop their full flavor. On the contrary for apples picked after the optimum harvest date storage ability is reduced. Establishing the proper time to harvest apple cultivars in relation to storage disorders and in order to ensure high quality after long-term CA storage requires special consideration. Post harvest ripening in apples is associated with color changes, softening of the fruit flesh, changes of the sour-sweet balance and a rise of ethylene. Several of these parameters have been used for determination of the optimum harvest date. Unfortunately these harvest indices have no predictive value. As aroma is an exponentially evaluating parameter during the early picking period, we have used the sensitivity of the described analytical procedure for prediction of optimum harvest date for storage apples. At regular intervals in the premature stage the sum of esters or butyl acetate (for faster analysis) is measured after 2 days of ripening in standard conditions. The optimum harvest time for storage apples can be predicted

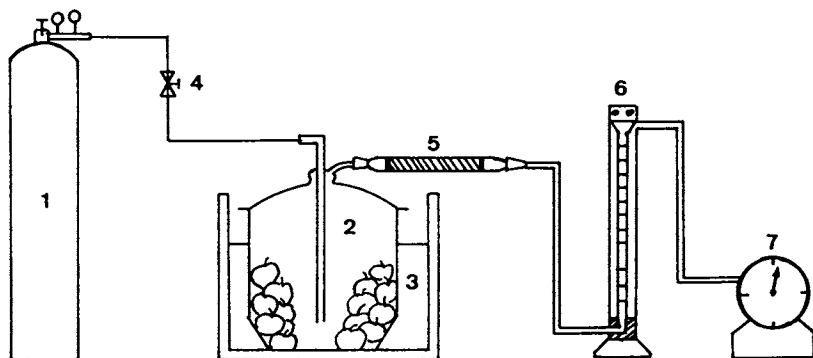


Figure 2. Apparatus for dynamic headspace sampling of volatiles, released from intact apples. 1 = high purity purge air, 2 = dessicators, 3 = thermostated waterbath (18°C), 4 = fine metering valve, 5 = Tenax adsorber, 6 = flow meter, 7 = wet-testmeter.

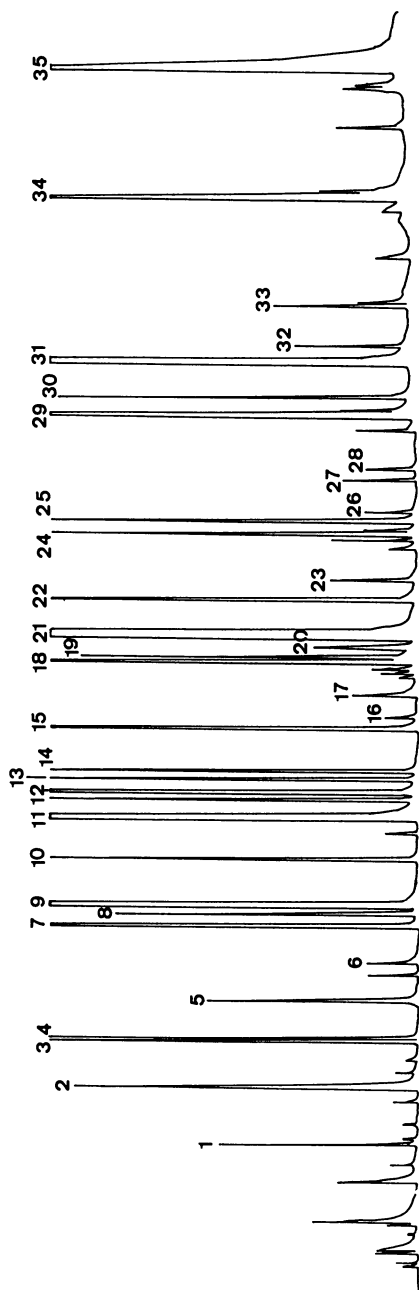


Figure 3. Chromatogram of the aroma of Spartan: 30mx0.5mm RSL-150 FS capillary column, linear temperature programming 20°C → 220°C, FID-detection. 1 = ethyl acetate, 2 = butanol, 3 = ethyl propionate, 4 = propyl acetate, 5 = 2-methylbutanol, 6 = 2-methylpropyl acetate, 7 = ethyl butanoate, 8 = propyl propionate, 9 = butyl acetate, 10 = ethyl 2-methylbutanoate, 11 = 2-methylbutyl acetate, 12 = propyl 2-butanoate, 13 = butyl propionate, 14 = pentyl acetate, 15 = propyl 2-methylbutanoate, 16 = 2-methylpropyl butanoate, 17 = benzaldehyde, 18 = butyl butanoate, 19 = ethyl hexanoate, 20 = pentyl propionate, 21 = hexyl acetate, 22 = butyl 2-methylbutanoate, 23 = 2-methylbutyl butanoate, 24 = propyl hexanoate, 25 = hexyl propionate, 26 = heptyl acetate, 27 = pentyl 2-methylbutanoate, 28 = hexyl 2-methylpropionate, 29 = butyl hexanoate + hexyl butanoate, 30 = 4-methoxyallylbenzene, 31 = hexyl 2-methylbutanoate, 32 = 2-methylbutyl hexanoate, 33 = hexyl pentanoate + pentyl hexanoate, 34 = hexyl hexanoate, 35 = α -farnesene.

by linear regression between the logarithm of the butyl acetate concentration and the picking date. The results for Golden Delicious apples in the season 1986 are presented in Figure 5. As evaluated by storage experiments and subsequent aroma analyses optimum harvest corresponds to a butyl acetate concentration of 0.4 $\mu\text{g}/\text{kg}$ apples/6 l dynamic headspace.

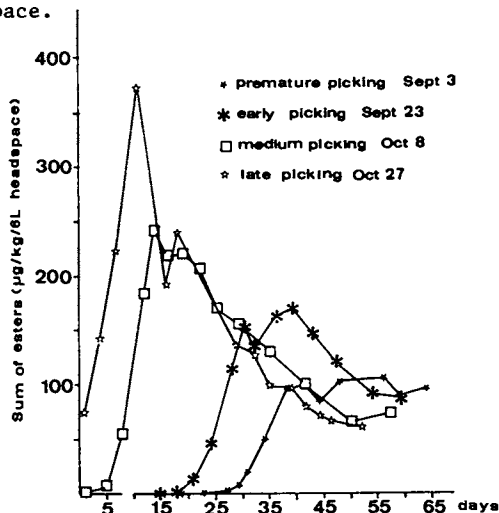


Figure 4. Influence of date of gathering on the evolution of the sum of Golden Delicious esters as a function of days of ripening in standard conditions.

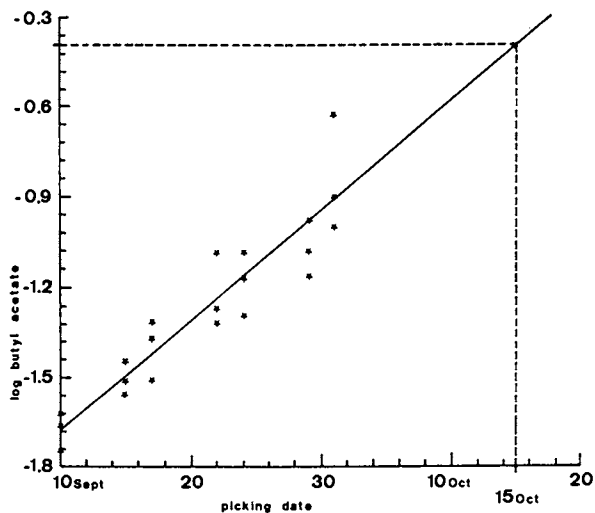


Figure 5. Prediction of the optimum harvest time for storage apples by linear regression between log butyl acetate (after 2 days of ripening) and picking date.

Postharvest Preservation Technology

In most countries an important part of the apple production is stored. Early studies showed that high carbon dioxide levels and low oxygen atmospheres could delay ripening. Choosing the storage circumstances seems largely to have been a question of trial and error and an impressive amount of work has been done about storage in high carbon dioxide and/or low oxygen atmospheres (20), under hypobaric or low ethylene conditions (21,22).

Aroma analysis can be used as an objective criterion for measurement of the evolution of flavor quality as a function of controlled-atmosphere storage time. Some years ago we measured the evolution of aroma development in Golden Delicious apples after removal from CA-storage for six periods during the storage period by means of a headspace technique, which isolated the volatiles released during maceration of the fruits (23). In a latter experiment we used the non-destructive headspace sampling technique with intact apples. Because the same fruits are sampled throughout the complete ripening experiment this technique has several advantages, such as convenience, faster analysis and better reproducibility. An illustration of the aroma development after removal from ventilated controlled atmosphere (Temperature : 0,5°C, 16% carbon dioxide, 5% oxygen) is shown in figure 6. Results objectively indicate an important decrease in aroma quality after long CA-storage.

Postharvest preservation technology is still in progress and more and more attention is given to low oxygen and to low carbon dioxide levels (ULO-storage) and to ethylene scrubbing. Our future research in this field will be directed to the evaluation of these recent developments in order to maintain flavor quality after long storage. The proposed technique can be used together with panel evaluations to compare different storage conditions.

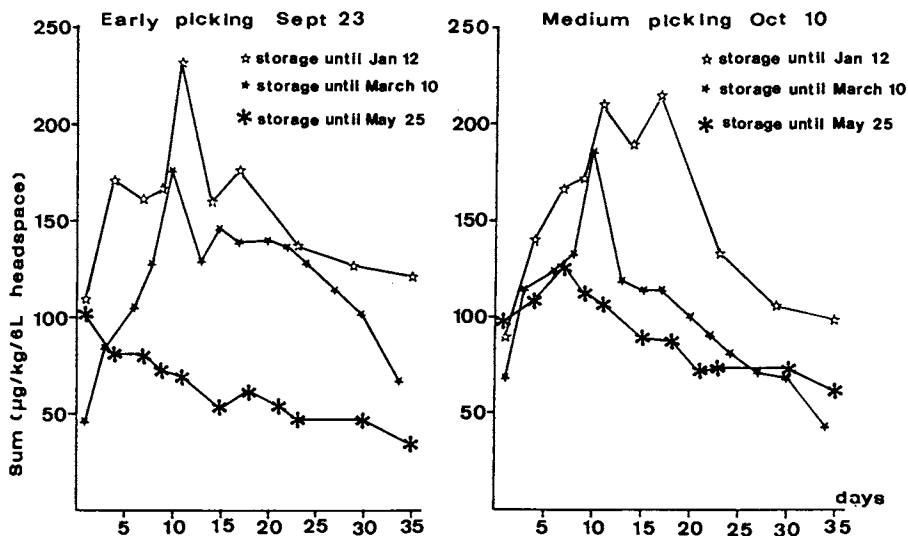


Figure 6. Aroma development after removal from ventilated controlled atmosphere. Influence of storage time and picking date.

Genetic Control : Cultivars

One of the important strategies of the recent quality policy of the apple industry consist in diversification of the number of cultivars, which are offered to the consumer. For instance the Belgian production, which around 1980 consisted of 75% Golden Delicious has been diversified to about 33% Golden Delicious, 24% Jonagold, 18% Boscoop, 6.5% Cox's Orange Pippin, 4.3% Gloster and others. As apple cultivars can differ markedly in aroma character, the decision which cultivars should be grown is of extreme economic importance. Classification of apple cultivars according to their aroma composition can be helpful for cultivar selection and for detection of relationships between cultivars. Information on the aroma patterns of different apple cultivars is scarce (24,25).

In a recent project we have determined the aroma development of 25 apple cultivars by means of dynamic headspace isolation of the volatiles released by intact fruits, followed by high resolution gas chromatography and mass spectrometric identification. Data acquisition resulted in 25 tables with quantitative data for about forty compounds at 5 to 8 ripening stages. For classification purposes the composition near the maximum of aroma development was used and 12 parameters were selected : hexanol, butyl acetate, hexyl acetate, butyl propionate, hexyl propionate, ethyl butanoate, propyl butanoate, butyl 2-methylbutanoate, hexyl 2-methylbutanoate, hexyl hexanoate, 4-methoxyallylbenzene and α -farnesene. Parameter selection was guided by visual comparison of the chromatogram and also some analytical and biochemical insights were taken in consideration. Hexanol was selected as a representative of alcohols, which are characteristic of some apple cultivars. The different esterified C₁ to C₆ acids were represented by the high concentration butyl and hexyl esters, except for the butanoates. As respectively butyl butanoate/ethyl hexanoate and hexyl butanoate/butyl hexanoate were not well separated on the RSL-150 (SE-52) capillary column, we used ethyl and propyl butanoate for representing the butanoates and disregarded butyl hexanoate. Furthermore 4-methoxyallyl-benzene, responsible for the spicy note in apples, and α -farnesene as the main sesquiterpene were used for classification.

Data processing was performed by Principal Component Analysis on a personal IBM computer. In a first set the procentual values of the selected parameters of the 25 apple cultivars were used. A projection of the 12-dimensional into a 2-dimensional space is presented in Figure 7. The reduced space presents in an optimal way (75.5% of the original variance) the relations between the objects. In the same plane the relations between the variables are given and can be related to the objects. Figure 7 shows that a large group of cultivars is characterised by high concentration of acetates. High concentrations of hexanol and ethyl butanoate are typical for another group, composed of Nico, Granny Smith, Summerred and Paulared. Boscoop and Jacques Label are outliers and are characterised respectively by high α -farnesene and high hexyl 2-methylbutanoate.

In a second data set the relationships between the closely related "acetate"-type cultivars was examined. The results for 17 cultivars are presented in a 3-dimensional space (88% of the original variance) in Figure 8. From this picture it is clear that f.i. Jonagold (Golden Delicious x Jonathan) en Golden Delicious have very

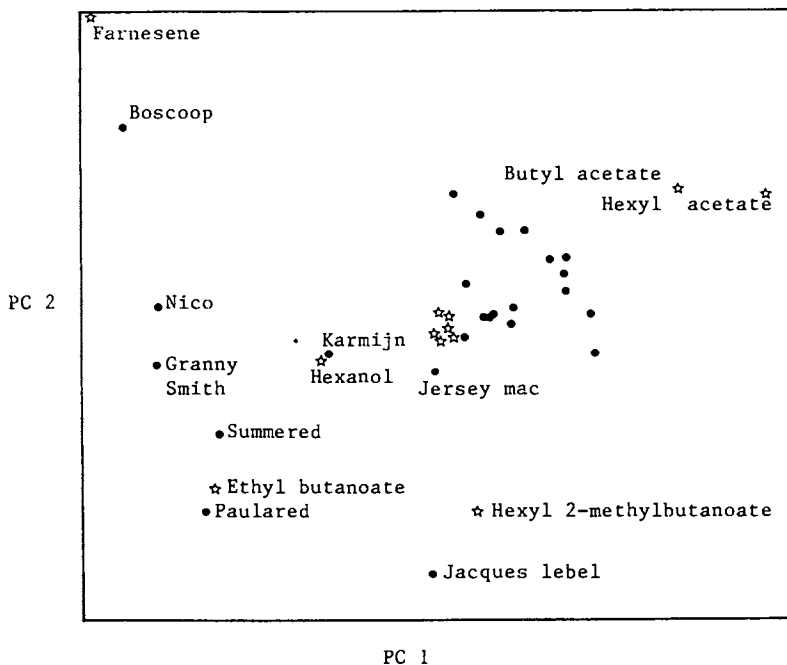


Figure 7. Principal component analysis of 25 apple cultivars.

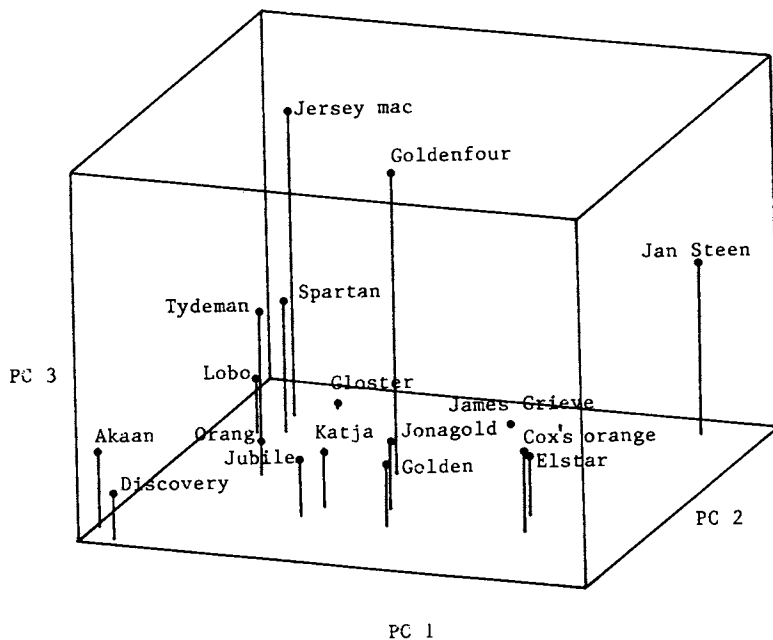


Figure 8. Principal component analysis of 17 "ester"-type apple cultivars.

similar aroma patterns. The results are especially of importance for cultivars with future potential for the apple industry, e.g. Elstar (Golden Delicious x Ingrid Marie), which has a similar aroma pattern as Cox's Orange Pippin.

Metabolic Control : Flavour Formation

Quantitative differences in volatile ester composition of different apple varieties may be explained by their biogenesis. In apples, the generation of aroma esters takes place mainly in the peel, is oxygen dependent and requires the organisation of intact tissue (5). In incorporation experiments apple disks were treated with carboxylic acids or aldehydes to study ester formation (26). As we thought that wounding of the tissues by preparing disks might disturb the normal behavior of fruits by dearranging the tissue organisation, intact apples were treated with aldehydes and carboxylic acids in the vapor phase (27,28), and volatiles were collected by dynamic headspace sampling on Tenax GC. Subsequent analysis gave a much more detailed picture of the aroma development compared to the direct headspace sampling in incorporation experiments (29,30). As an illustration of the effect of adding aldehydes, Figure 9 shows a graphical picture of the volatiles, isolated by dynamic headspace sampling 1 day after treatment of Golden Delicious apples with pentanal and hexanal. Treatment of intact fruits with carboxylic acids or aldehydes led to the enhanced formation of volatile esters and showed that the fruits possessed activity for esterification, for α - and β -oxidation, for aldehyde reduction, and also were capable of reducing carboxylic acids into alcohols probably by the way of the corresponding aldehydes (31,32).

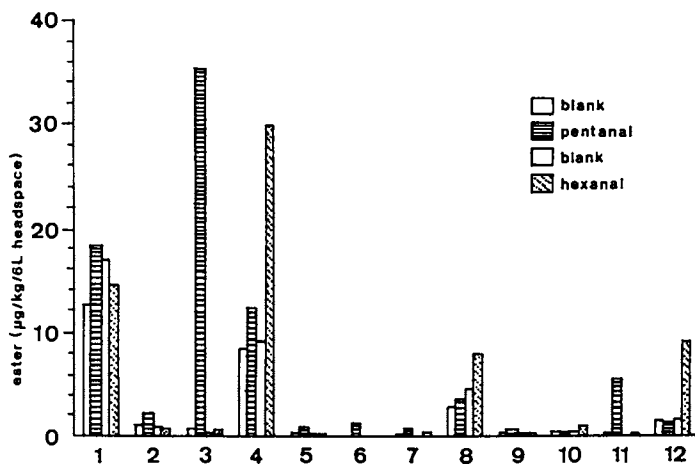


Figure 9. Comparison of ester formation by Golden Delicious apples one day after treatment with pentanal and hexanal. 1 = butyl acetate, 2 = 3-methylbutyl acetate, 3 = pentyl acetate, 4 = hexyl acetate, 5 = butyl propionate, 6 = pentyl propionate, 7 = hexyl propionate, 8 = butyl butanoate, 9 = butyl 2-methylbutanoate, 10 = hexyl 2-methylbutanoate, 11 = hexyl pentanoate, 12 = hexyl hexanoate.

Conclusions

Aroma development in ripening apples is dependent on a number of external and internal factors. Dynamic headspace sampling of the volatiles from intact fruits, followed by high resolution gas chromatography or g.c.-m.s. analysis is a convenient procedure for the study of these factors.

Acknowledgment

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Chapter 4

Nonvolatile Conjugates of Secondary Metabolites as Precursors of Varietal Grape Flavor Components

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An examination of hydrolysates produced by glycosidase enzyme or pH 3.2 acid treatment of C₁₈ reversed-phase isolates from juices of "non-floral" Vitis vinifera vars. Chardonnay, Sauvignon Blanc and Semillon demonstrated that these grapes contain conjugated forms of monoterpenes, C₁₃ norisoprenoids, and shikimic acid-derived metabolites. The volatile compounds obtained hydrolytically from the conjugates were produced in sufficient concentration to permit ready analysis by GC/MS. The products of pH 3.2 hydrolysis have sensory significance when assessed in a neutral wine. The study further develops the precursor analysis approach as a technique to facilitate research into varietally specific constituents of grapes.

The most extensively studied flavor compounds of Vitis vinifera grapes are monoterpenes (1). These compounds, along with other well recognized constituents of grape berries such as anthocyanins, hydroxycinnamate esters and tannins, are categorized by plant biochemists as secondary metabolites. One of the defining characteristics of secondary metabolites is their propensity to accumulate in particular organs or tissues of the host (2). This accumulation occurs presumably either as a result of products being formed in terminating biosynthetic pathways, or because of relatively slow product turnover. However, in the case of grape monoterpene flavor compounds, as with the flavor constituents of most other fruits, experience proves that accumulation is not one of their significant properties. Flavor compounds of grapes are trace constituents only, and even in berries of the most floral varieties, i.e. the Muscats, monoterpene flavor compounds are present at concentrations of only 1-2 mg/L of juice (3).

Research over the last few years has revealed that the flavor compounds are not end products of monoterpene biosynthesis in the grape. Oxidative pathways, leading to flavorless polyhydroxylated forms of the monoterpenes, are active in Vitis vinifera. Also, the

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grape's monoterpenes, in both unoxidized forms and as polyols, occur as glycosidic conjugates in the fruit (1). More recent evidence (4) indicates that glycosylation takes place after the extra hydroxyl groups are introduced into the monoterpene skeleton, which is consistent with the view that glycosylation is usually the terminal step of any biosynthetic pathway (5). In keeping with this proposal analyses have shown that, for most monoterpene dependent varieties, the glycosides accumulate in the berries to a greater extent than the free compounds (3, 4).

A second property of many of the monoterpene glycosides is their high reactivity in weakly acid media (1). Acid-catalyzed hydrolysis at pH 3.0-3.5 of these non-volatile and flavorless glycosidic compounds, can give volatile monoterpenes, some of which have significant sensory properties. Accordingly, the presence of many monoterpenes found in grapes and wines can be accounted for by non-enzymatic reactions (1).

Thus, two diametrically opposite consequences follow from these secondary transformations of the grape's monoterpenes. Conjugation of monoterpenes by glycosylation is a process diminishing the pool of free compounds and thereby denudes the fruit of flavor. Simultaneously, however, some of the conjugates are activated by glycosylation and can thus regenerate volatiles, different from the aglycons, by non-enzymatic mechanisms.

Recognition of these phenomena in grapes and wines (1), and the importance therefore of the glycosides as precursors of flavor in those systems (6-8) has stimulated much research interest in the role of non-volatile flavor precursors in other fruits (9-11), processed fruits (12), and leaf products (13, 14). For convenience this line of investigation is described here as the "precursor analysis approach" to flavor research.

This paper discusses new developments in this field of research and in particular the application of the precursor analysis approach to the study of flavor compounds of non-monoterpene containing grape varieties.

Glycosylation and the Flavor Properties of Non-Floral Grape Varieties

From observations of the monoterpene aglycons enzymatically released from the glycosidic fractions of different floral varieties, it was concluded that glycosylation was a pathway by which the grape "fixes" the monoterpene alcohol composition (15). Thus, analysis of aglycons can furnish a metabolic fingerprint for the monoterpenes of a particular variety, indicating *inter alia*, the extent of accumulation of individual compounds as glycosides, the degree of hydroxylation of the terpenes, and the type of products formed by oxidation. This interpretation has, up until now, been assumed to be limited to that relatively small number of floral grapes which are dependent on monoterpenes for their varietal flavor (6).

In non-monoterpene dependent grape varieties the flavor is often delicate and subtle, and knowledge of the chemical composition of the flavor compounds is almost non-existent. A notable exception in this regard is the recent confirmation of the role of alkyl methoxypyrazines in contributing to the varietal character of Sauvignon Blanc wines (16, 17). However, for other grapes in this

non-monoterpene dependent category, which incidentally make up the majority of the world's winemaking varieties, even basic information on the types of compounds which might be contributing to the characteristic flavors is not available.

The major reason for this deficiency is the tiny quantity of volatiles present in juices of these non-floral grapes. Thus, in spite of constant advances being made in the techniques of GC/MS for the identification of flavor compounds (18) there has been little progress in this area of varietal grape flavor research. Accordingly, it was clear that an alternative approach to the problem should be sought.

To this end a strategy was developed based on the hypothesis that similar metabolic processes operate on the unknown flavor compounds of non-floral varieties to those which transform the monoterpenes of the floral grapes, i.e. oxidative hydroxylation of some compounds, followed by conversion of these, and of unoxidized volatiles, to flavorless glycosides. Additionally, it was assumed that glycosylation would 'fix' the composition of the alcohols and related derivatives and allow them to accumulate in a manner similar to that observed for the monoterpene aglycons. Accordingly, analysis of the glycoside fractions of non-floral grapes, i.e. precursor analysis, could lead not only to a rationalization of those volatiles formed by non-enzymatic steps in these fruits, but also to an elucidation of the free impact flavor compounds from which the conjugates were formed.

The Precursor Fractions of Sauvignon Blanc, Semillon and Chardonnay Grapes

The C_{18} reversed-phase (RP) liquid chromatographic procedure used for isolation of monoterpene glycosides from floral varieties (19), was applied to juices of three non-floral grapes, i.e. Sauvignon Blanc, Semillon and Chardonnay. Hydrolysis of the isolated fractions, both enzymatically and by aqueous acid at pH 3.2, gave a range of volatiles (see Table I) confirming that these grapes also accumulate low molecular weight compounds as conjugated derivatives. Acid hydrolyses were carried out by heating anaerobically the C_{18} RP isolates from 4.5L of juice at 50° for 1 month in 90mL of aqueous saturated potassium hydrogen tartrate, adjusted to pH 3.2 with tartaric acid.

Sensory Significance of the Precursor Fractions and Their Hydrolysates

Before progressing further with the chemical analysis of the hydrolysates, the sensory significance of these and of the C_{18} RP precursor materials was assessed.

Samples of the unhydrolysed C_{18} RP concentrates, the enzyme released aglycons, and the pH 3.2 acid hydrolysates from each grape variety, were presented to a panel for duo-trio aroma assessment in a neutral wine medium. Untreated fractions and aglycons were presented for evaluation at concentrations equivalent to twice that in the original juices, and the acid hydrolysates were presented at 1.5 times their original juice concentrations. Other conditions used for

Table I. Aglycons and pH 3.2 Hydrolysis Products from C₁₈ RP Isolates

Compound ^a	Sauv. Blanc		Chardonnay		Semillon		Evid ₅ ence	Ref
	Agly	H ⁺	Agly	H ⁺	Agly	H ⁺		
MONOTERPENES								
Furan linalool oxide 1 1		++		+++		++	A	1
Furan linalool oxide 2 1		++		++		++	A	1
Hotrienol 2		+				++	A	1
Nerol oxide 3		+				+	A	1
α-Terpineol 4	++						A	1
A pyran linalool oxide 5				+	++	+	A	1
2,6-Dimethylocta-3,7-diene-2,6-diol 6	++	++		+	++	++	A	1
Geraniol 7	++				++		A	1
2,6-Dimethyloct-7-ene-2,6-diol 8		+++		++		+++	A	1
cis-1,8-Terpin 9		+++		++		+++	A	1
trans-1,8-Terpin 10		+++		++		++	A	1
(Z)-2,6-Dimethylocta-2,7-diene-1,6-diol 11	++			+		++	A	4
(E)-2,6-Dimethylocta-2,7-diene-1,6-diol 12	+++			++		+++	A	4
Unknown paramenthenediol 1		++					B	
Unknown paramenthenediol 2		+		+			B	21
Unknown paramenthenediol 3	++++	+				++	B	21
NORISOPRENOIDS								
6-Methylhept-5-en-2-one	++						B	22
2,6,6,-Trimethylcyclohex-2-ene-1,4-dione		++		++		++	C	23
Vitispiranes 13		++		++		++	A	24
2,2,6-Trimethylcyclohexane-1,4-dione				+		+	A	25
1,1,6-Trimethyl-1,2-dihydronaphthalene 14		+		++		+	A	19
Damascenone 15		++		++		++	C	19
Actinidol 1 16		++		+++		+++	A	26
Actinidol 2 16		+++		+++		+++	A	26

Table I. Continued

Compound ^a	Sauv. Blanc		Chardonnay		Semillon		Evid ^b ence	Ref
	Agly	H ⁺	Agly	H ⁺	Agly	H ⁺		
Actinidol 3 17				+		++	B	26
2-(3-Hydroxybut-1-enyl)- 2,6,6-trimethylcyclohex- 3-en-1-ones 18		+++		+++		+++	A	27
3,4-Dihydro-3-oxo- actinidol 19				++			A	25
3-Oxo- β -damascone 20	+++		++				E	21
3-Hydroxy- β -damascone 21	++	+	+	++	+	++	C	21
3,9-Dihydroxymegastigm- 5-en-7-yne 22	++		++		+		E	28
3-Oxo- α -damascone 23					+		E	21
3-Oxo- α -ionol 24 ^c	+++	+	++	+++	+++	++	C	25
3-Oxo- α -ionone 25				+		+	C	25
9-Hydroxymegastigma-4,6- dien-3-one (isomer 1) 26				++			E	29
Blumenol C 27	+++		+		+		E	30
9-Hydroxymegastigma-4,6- dien-3-one (isomer 2) ^d 26				++			E	29
Unknown A (isomer 1) ^d	++				++		B	21
Unknown A _e (isomer 2) ^d	+++	++	++	+++	+		B	21
Unknown B ^e	+++		+++		+++		B	21
Vomifoliol 28	+++	++	+++	+++	+++	+++	C	25
Dehydrovomifoliol 29	++	+		++	+	++	C	25
SHIKIMATE-DERIVED								
Benzaldehyde		++		++		++	C	
Benzyl alcohol	++++	+++	++	++	+++	+++	A	31
2-Phenylethanol	+++	+++	++	+++	++	+++	A	31
2-Hydroxybenzoic acid methyl ester				+		+	B	
Benzoic acid		++		+++	+	++	C	
Phenylacetic acid				++		++	C	
2,3-Dihydroxybenzoic acid methyl ester	+						C	
Vanillin 30	+	++		+++		++	C	21
Methyl vanillate 31			+	+	+	+	C	21
Acetovanillone 32	+			++	+	++	D	22
4-Hydroxybenzaldehyde		+++		+++		+	C	
Tyrosol 33	++		+		++	++	D	22
Benzophenone		++					D	22
4-Hydroxybenzoic acid methyl ester				++			D	22
4-Hydroxyacetophenone		++		++		+	C	

Continued on next page.

Table I. Continued

Compound ^a	Sauv. Blanc		Chardonnay		Semillon		Evid _b ence	Ref
	Agly	H ⁺	Agly	H ⁺	Agly	H ⁺		
2,5-Dihydroxybenzoic acid methyl ester	+++	+	+	+	+		C	
Vanillic acid 34				++			D	22
Raspberry ketone 35					++	++	D	22
Zingerone 36					+		A	21
Dihydroconiferyl alcohol 37			+	+	+		A	21
Benzyl benzoate		+				+	B	
Syringaldehyde 38		+		+			D	22
4-Hydroxybenzoic acid				+++			D	22
Methyl syringate 39				++			B	
Acetosyringone 40				+			D	22
Coniferyl alcohol 41			+		+		A	21
Syringic acid 42				++			D	22

Constituents in each class are listed in order of increasing retention time on a J&W DB 1701 fused silica capillary G.C. column. Quantities were estimated by a comparison of the total ion count for the mass spectrum of each peak with that of an n-octanol internal standard; +, 0.1-1 µg/L; ++, 1-10 µg/L; +++, 10-100 µg/L; +++, 100-500 µg/L. ^a For compound structures see Figure 1. ^b A, previously identified in this laboratory; B, tentatively identified from interpretation of the mass spectrum; C, symmetrical peak enhancement and identical mass spectrum on coinjection with an authentic sample; D, comparison of mass spectrum with published spectrum; E, comparison of mass spectrum and retention time with that of material available commercially or synthesized in the laboratory. ^c Both diastereoisomers observed in each of the acid hydrolysates, only one in each of the aglycon fractions. ^d This is unknown 17 of Ref. 21. ^e This is unknown 16 of Ref. 21.

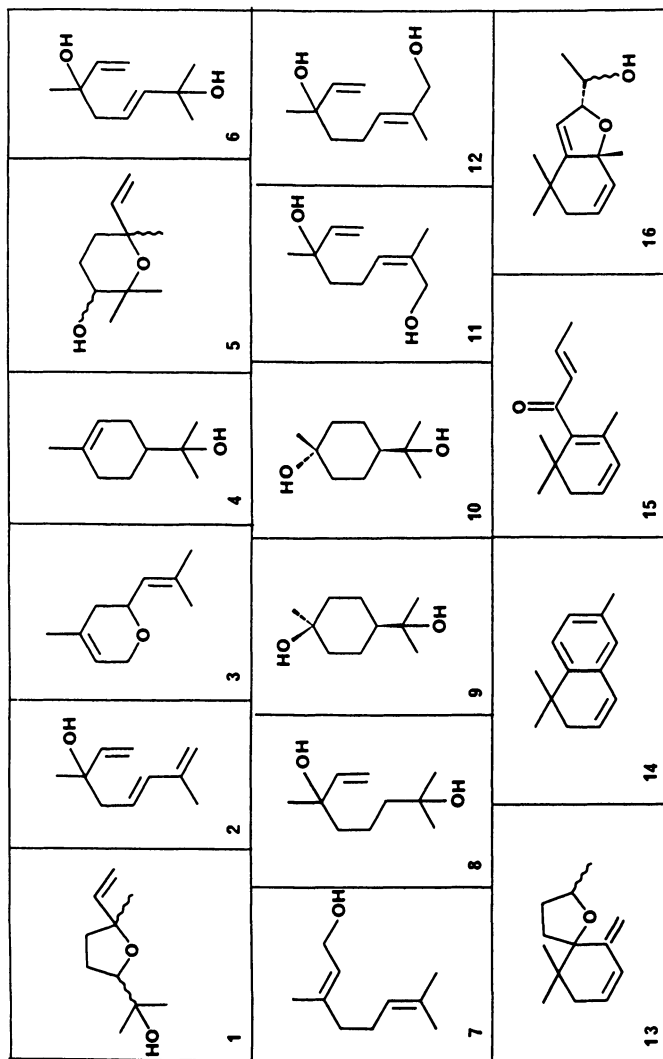


Figure 1. Some compounds referred to in this work.

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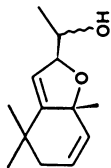
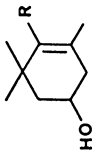
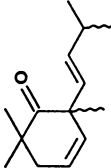
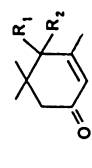
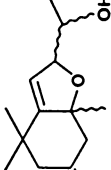
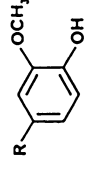
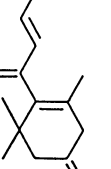
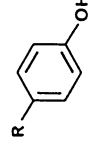
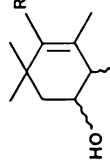
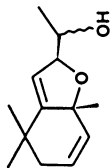
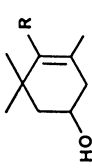
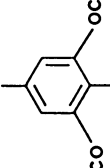
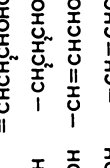
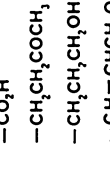
 <p>17</p>	 <p>21</p> <p>22</p> <p> $\frac{R}{-COCH=CHCH_3}$ $-C\equiv CCHOHCH_3$ </p>	 <p>18</p>	 <p>23</p> <p>24</p> <p>25</p> <p> $\frac{R_1}{H}$ $\frac{R_2}{-COCH=CHCH_3}$ $-CH=CHCHOHCH_3$ $-CH=CHCOCH_3$ </p>	 <p>19</p>	 <p>30</p> <p>31</p> <p>32</p> <p>34</p> <p> $\frac{R}{-CHO}$ $-CO_2CH_3$ $-COCH_3$ $-CO_2H$ </p>	 <p>20</p>	 <p>33</p> <p>35</p> <p> $\frac{R}{-CH_2CH_2OH}$ $-CH_2CH_2COCH_3$ </p>	 <p>43</p> <p>44</p> <p> $\frac{R}{-CH_2CH_2CHOHCH_3}$ $-CH=CHCHOHCH_3$ </p>
 <p>38</p> <p>39</p> <p>40</p> <p>42</p> <p> $\frac{R}{-CHO}$ $-CO_2CH_3$ $-COCH_3$ $-CO_2H$ </p>	 <p>26</p> <p>27</p> <p>28</p> <p>29</p> <p> $\frac{R_1}{H}$ $\frac{R_2}{-COCH=CHCH_3}$ $-CH=CHCHOHCH_3$ $-CH=CHCOCH_3$ </p>	 <p>36</p> <p>37</p> <p>41</p> <p> $\frac{R}{-CHO}$ $-CO_2CH_3$ $-COCH_3$ $-CO_2H$ </p>	 <p>33</p> <p>35</p> <p> $\frac{R}{-CH_2CH_2OH}$ $-CH_2CH_2COCH_3$ </p>	 <p>43</p> <p>44</p> <p> $\frac{R}{-CH_2CH_2CHOHCH_3}$ $-CH=CHCHOHCH_3$ </p>				

Figure 1. Continued.

the sensory studies have been described previously (20). Results of these experiments are given in Table II.

Table II. Summary of Sensory Panel Responses for Duo-Trio Aroma Evaluations of Glycosidase Enzyme Hydrolysates, pH 3.2 Acid Hydrolysates, and Untreated C₁₈ RP Fractions From Three Non-Floral Grape Varieties

Variety	Significance of Responses		
	Untreated ^a	Enz. Hydrol. ^a	Acid Hydrol. ^b
Sauvignon Blanc	NS	*	**
Chardonnay	NS	NS	***
Semillon	NS	NS	***

NS = not significant, * = significant at 5%, ** = significant at 1%, *** = significant at 0.1%.

a, n = 13 judges; b, n = 16 judges

It is clear from the data in Table II that the acid hydrolysates of the precursor fractions had a highly significant effect on the aroma of the wines to which they were added. In contrast, neither the original precursor materials nor the glycosidase-released aglycons, with the possible exception of those from Sauvignon Blanc, could be detected by the panel.

These observations are in accord with the results of a previous study which demonstrated that the aglycons from a Chardonnay glycosidic fraction were not a significant source of added aroma to the wine from which it had been obtained (20). The present work emphasises the importance of mild acid hydrolysis and the associated chemical pathways to yield flavor significant volatiles in these non-floral varieties.

Sensory descriptive analyses of the acid hydrolysates are in progress, and preliminary data from these experiments for the products given by the Chardonnay precursors at pH 3.2 indicate that oak wood and vanilla descriptors are important to the sample of this variety.

Chemical Composition of the Precursor Fraction Hydrolysates

It can be seen from the data in Table I that the aglycons and the products given by mild acid treatment of the C₁₈ RP retained material from the three grape varieties were made up of monoterpenes, thirteen-carbon and other norisoprenoids, together with a group of shikimate-derived aromatic compounds. It is also apparent that the acid hydrolysates were more complex than the respective aglycon fractions, with the acid-derived products containing many compounds not seen in the material released by the glycosidase enzyme.

In addition to the products listed in Table I, a large number of, as yet, unidentified compounds was found, mainly in the acid hydrolysates. Of this group, five were monoterpenes, twenty-one were norisoprenoid and twenty-three were in the aromatic or phenolic class. The unknown norisoprenoids, aromatic and

phenolic compounds, were observed predominantly in the Chardonnay and Semillon hydrolysates. At least six nitrogen-containing compounds and two γ -lactones were also observed, together with a large number of minor constituents for which no structural category has yet been assigned.

The Monoterpenes. Monoterpenes are not major components of non-floral grapes (1) although some of these compounds were present in each of the three varieties. In a previous survey of monoterpene diols in floral and non-floral grapes, (Z)- and (E)-2,6-dimethylocta-2,7-diene-1,6-diols 11 and 12 were not found in the Chardonnay samples examined (4). Observation of these diols in the Chardonnay here, and reports of their occurrence in several other Chardonnay clones (7), indicates that these compounds 11 and 12 are common monoterpenes of grapes. No products obviously derivable from a conjugate of diols 11 and 12 were seen in the acid hydrolysates, which is consistent with the previously reported stability of the 1,6-diol system in these compounds (4).

The 1,8-terpins 9 and 10, which are monoterpenes of high flavor threshold, were observed in the acid hydrolysates of each variety. These diols are derivable as thermodynamically stable end-products of acid catalyzed rearrangement of monoterpenes which are at the oxidation state of geraniol. However, few compounds at this oxidation state were seen among the aglycons, and the precursors of the terpins 9 and 10 in these varieties have yet to be discovered.

The Norisoprenoid Compounds. The C_{12} -norisoprenoid compounds in Table I can be grouped into four different oxidation levels. Compounds exemplifying these four levels are vitispirane 13, damascenone 15, vomifoliol 28, and dehydrovomifoliol 29. Such subclassification should help in establishing the nexus between hydrolysis product and precursor. Nevertheless, many of the compounds given by acid hydrolysis cannot yet be related directly back to the aglycons observed. Thus, for example, previous research has shown that megastigmatriols 43 and 44, which occur naturally in grapes, give on hydrolysis several of the norisoprenoids observed in this study, i.e. vitispirane 13 from 43, and the actinidols 16 and 17 together with hydrocarbon 14 and the rearranged ketone 18 from 44 (27). However, neither triols 43 and 44, nor any obviously related isomers, were observed among the aglycons, and precursors of volatiles 13, 14, 16, 17 and 18 have yet to be found in these non-floral varieties.

Conversely, many of the major norisoprenoid aglycons recorded in Table I do not appear to contribute significantly to the products given by acid hydrolysis. The hydrolytic chemistry of vomifoliol 28 and 3-oxo- α -ionol 24 has been studied (25); only trace amounts of 3-oxo- α -ionone 25 and an isomer of 3,4-dihydro-3-oxoactinidol 19, which are derivable from the former aglycon 28, were observed, and none of the products reported (25) from 3-oxo- α -ionol 24 were found.

Obviously further work is needed in linking the norisoprenoid components of the precursor fractions to volatiles in the hydrolysates. An important aspect of this will be the elucidation of the transposition mechanism of the oxygen function from position

9 to 7 in the megastigmane skeleton, with the formation of damascenone 15 and relatives 20 and 23.

The Shikimate-Derived Group. Examination of the aromatic compounds seen here and previously (21) indicate that C₆-C₁, -C₂, -C₃ and -C₄ compounds, showing a variety of hydroxy- and methoxy-substitution patterns, are present in the grape precursor fractions. These products are known to be derived in plants from phenylpropanoids via side-chain degradation and elongation reactions (32, 33). Because of the obvious sensory significance of many of these aromatic compounds, e.g. vanillin 30, raspberry ketone 35 and zingerone 36, the incidence of occurrence, and the origins of individual constituents within this class of secondary metabolite, together with the nature of the conjugating moieties involved with them, are now recognized as subjects of high importance to grape flavor.

Compositional Differences Among the Varieties

Differences among the aglycons and acid hydrolysates of these three non-floral varieties would not be expected to be as obvious as between those of floral and non-floral grapes (15). Nevertheless, this first exploratory investigation into the hydrolytically produced volatiles from the C₁₈ RP precursor fractions of Chardonnay, Semillon and Sauvignon Blanc allows differences to be discerned. These are best assessed by considering each of the three groups of compounds individually.

From Table I it can be seen that Chardonnay contained fewer and lower concentrations of monoterpenes than either the Semillon or Sauvignon Blanc juices. With regard to the norisoprenoid compounds, the Sauvignon Blanc juice appeared to contain a higher concentration of these constituents in glycosidically bound form than did the Chardonnay or Semillon juices. In contrast the C₁₈ RP fractions from the latter varieties yielded norisoprenoids, including the unknowns in this group, in greater abundance and variety following pH 3.2 hydrolysis than did the Sauvignon Blanc sample. More phenolic and aromatic constituents were yielded by the Chardonnay precursor fraction than from the other two varieties. Close examination of the phenolic and aromatic group of compounds suggests further distinguishing features. For example, while C₆-C₁ and C₆-C₂ compounds were common to all of the grapes, Chardonnay and Semillon appeared to produce greater amounts of aromatic compounds with longer side-chains than did the Sauvignon Blanc. The aromatic substitution patterns may also be influenced by varietal genetics. Thus guaiacyl-substituted compounds were observed mostly in Chardonnay and Semillon, while the more heavily substituted syringyl constituents occurred almost exclusively in the Chardonnay hydrolysates.

Conclusion

Previous work on precursor fractions from grapes had indicated that analysis of the conjugated constituents would facilitate research in the varietal specific constituents of fruit (21). This study further develops that research and proves that conjugated forms of

many secondary metabolites are present in the C₁₈ RP fractions from non-floral grapes and mild acid hydrolysis of these gave a number of volatiles, many of which are of known flavor importance. Furthermore, the acid hydrolysates from the three varieties were demonstrated to have sensory significance in wine. The research also indicates that differences in composition among the acid- and glycosidase enzyme hydrolysates of the three juice samples will allow varietal differences to be observed.

Future work must investigate the conjugating moieties involved with the grape secondary metabolites. Plants are known to employ sugars with both ester and glycosidic linkages, as well as several non-sugar moieties for conjugation purposes (34). Thus considering glycosides alone may not give a complete picture of the bound flavor compounds of the fruit. This is emphasized by the data in Table I in which many of the acid hydrolysis products cannot be rationalized in terms of the aglycons released by Rohapect C from the precursor fractions.

It will also be necessary to relate the structures of the conjugates in the precursor fractions with the composition of the hydrolysates. This is particularly important where polyfunctional molecules are involved allowing more than one site of conjugation, because acid hydrolysis of one conjugate of a particular secondary metabolite may yield different products from that of an alternatively or polyconjugated species.

Finally, and most importantly, the acid hydrolysates and enzyme deconjugated products can be used to facilitate investigations into those trace volatiles which are free in the grape or finished wine, and which are responsible for the varietal flavor.

The precursor analysis should now be seen as a useful complement to traditional methods of flavor analysis of fruits. The latter methods are often limited to the painstaking processes of isolation and identification of those trace constituents which are directly responsible for flavor. The precursor analysis approach takes advantage of the evidence provided by Nature when secondary metabolites, including flavor compounds, are biochemically transformed and accumulated by the fruit.

Acknowledgments

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Chapter 5

Sotolon

Identification, Formation, and Effect on Flavor

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Sotolon, 4,5-dimethyl-3-hydroxy-2(5H)-furanone, was isolated and identified as a flavor impact compound from raw cane sugar and has a very low threshold value. The aroma characteristic changes from caramel-like at low concentrations to curry-like aroma at high concentrations. For this reason the compound, which had already been synthesized and also found in its natural form, was not associated with the characteristic brown lump sugar aroma. The syntheses of sotolon homologues and their enantiomers provided some information about the structure-aroma relationship. The formation of sotolon was confirmed in a model system composed of glutamic acid and pyruvate, the latter being estimated as a reaction product of an amino-carbonyl reaction. As a flavor impact compound, sotolon was found in botrytized wine and roasted tobacco. The presence of sotolon in these products is indispensable for a flavor with high sensory qualities.

Cane sugar is one of the oldest agricultural products known to man and originated in the tropics. Despite the primitive technology for sugar processing that was used in the early stage of its development, raw cane sugar became edible and even palatable because of its acceptable flavor. Thus cane sugar history contrasts with the development and manufacture of beet sugar produced in northern Europe since the 19th century.

Brown lump sugar is prepared from calcified cane juice simply by boiling down into a solid form, which is widely used as an ingredient of traditional Japanese cakes in addition to other partially refined raw sugars. These are not only used as sweeteners but also as flavoring ingredients. The characteristic caramel-like, burnt-sweet aroma of raw cane sugar is known to be formed at the last stage of the heating and condensing process for sugar cane juice, and the flavor impact compound (FIC) has been estimated to be a nonenzymatic product formed during the browning reaction. Since

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Takei *et al.*(1) first reported in 1936 the separation and identification of a volatile component in raw cane sugar and molasses, many researchers have tried to identify the FIC of the characteristic sugary aroma in cane juice or raw cane sugar products. Furfural, hydroxymethyl furfural, maltol, isomaltol and 3-methyl-2-hydroxy-2-cyclopenten-1-one (cyclotene) have been identified as typical amino-carbonyl reaction products with a burnt sugary aroma; however, their threshold values are too high to explain the strong characteristic aroma of raw cane sugar. Through many research studies it has become clear that this sugary aroma could be concentrated in a specific fraction, which implies that one or a few FICs must be present, although the amount of FICs has been too little to identify by the usual analytical method.

Separation and Identification

As the content of the aroma substance was estimated to be extremely low, we used cane molasses as the starting material for our study, as the sugary aroma was already condensed in it and the material could be supplied in bulk by the manufacturer. One ton of cane molasses was first extracted with acetone, and after evaporating the solvent, the low molecular weight organic materials were extracted continuously with ether. The extract was then divided into basic, acidic, weakly acidic and neutral fractions; by an organoleptic evaluation the sugary aroma appeared strongly in both the weakly acidic and neutral fractions. As the yield of the neutral fraction was much higher than that of the other fractions, the combined weakly acidic and neutral fractions were further fractionated by silica gel column chromatography. The aroma was concentrated in fraction 11, which was then separated by packed-column GC with peak sniffing ("nasal appraisal"). At a retention time of about 60 min, the strong sugary aroma was noted, although many peaks overlapped in this zone. In the next step, the effluent between t_R 55 and 65 min was trapped repeatedly and analysed by high-resolution gas chromatography (HRGC) combined with mass spectrometry (MS). This separation scheme is summarized in Figure 1, (2,3) and the resolution of the GC trapping fraction by HRGC is shown in Figure 2.

By GC-MS analysis, peaks 36, 37 and 39 were estimated to be 3-hydroxy-4,5-dimethyl-2(5H)-furanone, acetate of hydroxymethyl-furfural and 4-pentyl-2-pentenolide, respectively. At this stage, the sample was too small to apply other analytical methods; therefore, we tried to synthesize all the possible compounds using the synthetic approaches described in the following section. None of the three synthetic products showed the characteristic sugary aroma that we had recognized in each separated fraction; however, the yield of fraction 11-GC TRAP from molasses was calculated to be ca. 1 ppm, and the concentration of FIC in molasses was estimated to be in the order of ppm or ppb from its peak area in the whole gas chromatogram. By diluting these synthetic products in water to the concentration of 1.0 ppm, 3-hydroxy-4,5-dimethyl-2(5H)-furanone

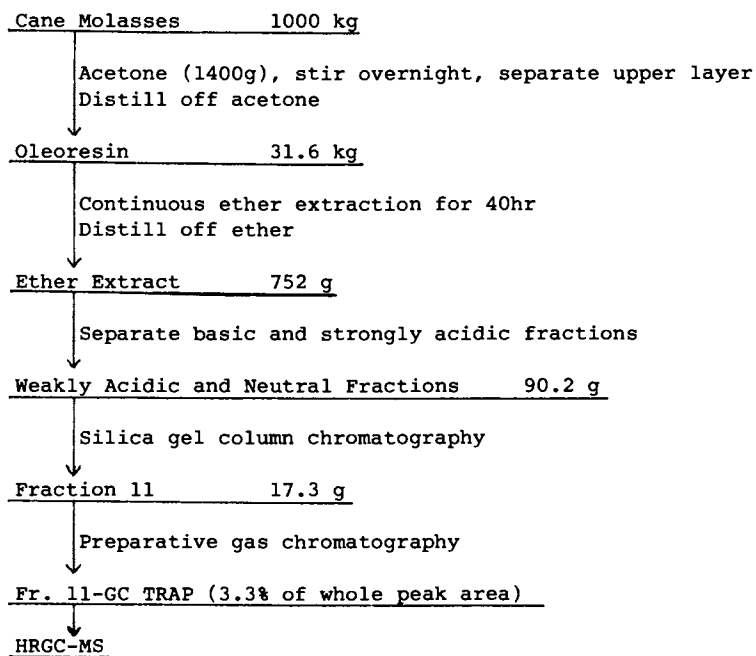


Figure 1. Scheme for Extraction and Fractionation

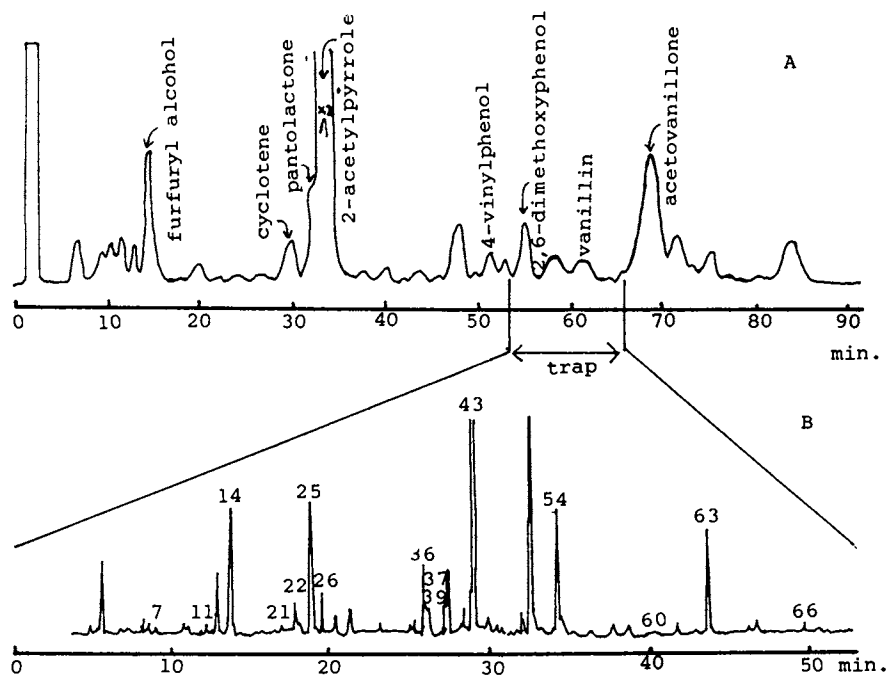


Figure 2. Gas Chromatographic Separation of the Aroma Components in Cane Molasses

(A) Preparative Gas Chromatography of Fr.11.

(B) Gas Chromatogram of Fr.11-GC TRAP (peak numbers in B correspond to those in Table 1.)

showed a strong burnt sugary aroma reminiscent of those in the separated fractions from molasses, all the gas-chromatographic and spectrometric data for the synthesized product coinciding with those for the natural one. Therefore, it became clear that a high concentration of the synthetic product produced another effect on the olfactory organ. In the next step to confirm the contribution to the sugary aroma in molasses, all the compounds identified in fraction 11-GC TRAP were reconstructed with the concentrations appearing on gas chromatogram B in Fig. 2, as is shown in Table 1, (A) without and (B) with sotolon.

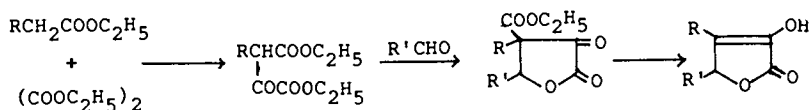
An organoleptic omission test on this mixture confirmed 3-hydroxy-4, 5-dimethyl-2(5H)-furanone to be the most important FIC for a raw cane sugar aroma, and the other main components in Table 1, i.e., vanillin, maltol, 4-pentyl butanolide, 4-vinyl phenol and 2,6-dimethoxy phenol, seemed to improve the overall sugary aroma. From these results, we(4) gave the trivial name "sotolon" to 2-hydroxy-4,5-dimethyl-2(5H)-furanone, which is built up from "soto" (raw sugar in Japanese) and "olon" (enol lactone) as a main FIC in raw cane sugar.

Table 1. The synthetic mixtures of the identified compounds in Fr. 11-GC trap

Peak No.	Compound	A Conc. (%)	B Conc. (%)
7	Furfurly alcohol	0.20	0.20
11	Damascenone	0.13	0.12
14	Guaiacol	0.72	0.69
21	5,6-Epoxy- β -ionone	0.04	0.04
21	Maltol	0.21	0.20
22	2-Acetylpyrrole	2.53	2.45
25	Phenol	5.70	5.51
26	4-Pentylbutanolide	3.38	3.27
36	Sotolon	-	3.27
37	5-Acetoxyethylfurfural	0.84	0.81
39	4-Pentyl-2-pentenolide	0.17	0.16
43	2,6-Dimethoxyphenol	67.12	64.92
50	Isoeugenol	0.17	0.17
54	4-Vinylphenol	1.90	1.84
60	5-Hydroxymethylfurfural	1.27	1.22
63	Vanillin	14.14	13.68
66	Acetovanillone	1.48	1.42

Synthetic Approaches

The synthesis of sotolon can be traced back to 1947(5). Sulser *et al.*(6) subsequently improved the synthetic method and obtained sotolon as a homolog of 3,4-dialkyl-2-hydroxy-butenolactone. They also identified II in a vegetable-protein hydrolysate as a flavoring compound(6), although they described the aroma character of I and II as Maggiherb-like at a concentration of 5-1 ppm, and as a walnut-like herbal aroma at 0.1 ppm with threshold values between 1-0.5 ppb. On the other hand, Rödel and Hempel(7) described the aroma character of I as fruity and alcoholic at the concentration of 100 ppm in water, and as a Maggi herbal and celery-like aroma at 50 ppm. We suggest the following reasons for there having been no description of a sugary aroma for synthetic I (sotolon): (a) The previous studies concerned the flavor substances of a vegetable protein hydrolysate, and when synthetic products showed a herbal aroma, they did not attempt an organoleptic test at lower concentrations. (b) These unsaturated lactones are unstable and easily polymerized to a viscous oil, although this change was often overlooked because of the strong odor. We, therefore, reinvestigated the synthesis of several sotolon homologs by following the general synthetic route described in Figure 3, and evaluated the aroma characters in their pure states at a concentration close to the respective threshold value. The structure and purity of distilled sotolon (bp 0.2 mm Hg 84-86°C) and the other homologs were confirmed by GC, MS, IR, PMR and CMR. The threshold values evaluated by an experienced panel from Takasago Perfumery Co. Ltd. are summarized in the bottom line of Figure 3 (unpublished data). In an earlier report (4), we assigned the threshold value of synthetic sotolon to be 0.002 ppb, although the new results have reduced this value to 10^{-3} ppb, and those of some longer alkyl-substituted homologues are ever lower. Sotolon is unstable in its pure state even in a refrigerator, although it can be preserved as a diluted solution in high polar solvents such as water, ethylene glycol or glycerin. These results suggest that sotolon is easily polymerized to a higher molecular weight product and thus gradually loses its strong aroma character.



	I	II	III	IV	V	VI	VII
R	Me	Me	Me	Me	Me	Et	Et
R'	Me	Et	Pro	Bu	iso-Bu	Me	Et
Threshold Value (ppb)	1×10^{-3}	1×10^{-5}	1×10^{-5}	5×10^{-6}	1×10^{-3}	2.5×10^{-4}	2.5×10^{-5}

Figure 3. General Synthetic Route to 3,4-Dialkyl-2-hydroxy-butenolactones and their Threshold Values

All the compounds produced a burnt sugary aroma, which became more burnt and heavy as the substituted alkyl chain increased in length. It is interesting that the ethyl substituted lactone II has a 100 times lower threshold value than that of sotolon, and that this compound has been considered to be an FIC in the protein hydrolysate. This was because it had been prepared from threonine by heating with hydrochloric acid and subsequent dehydration, hydrolysis, condensation (Aldol type) and decarboxylation, and it showed a strong curry-like or herbal aroma at concentrations higher than 1 ppm.

Sotolon has an asymmetric carbon in its molecule, and therefore, stereospecific syntheses(8) of sotolon enantiomers would be effective for correlating the stereostructure and olfactory sensation. If the aroma character turned out to be quantitatively or qualitatively different between these enantiomers, we could expect to elucidate whether sotolon is a naturally occurring compound or a product formed by chemical reaction during the sugar manufacturing process. Starting from D- and L-tartaric acids, the respective 2,3-epoxybutenes, (2R,3R) and (2S,3S), were prepared by the known method. 1,3-Dithiane-2-carboxylic acid condensed with the respective epoxides and removal of the thioacetal group gave optically active sotolones. The $[\alpha]_D^{23.5}$ value in ether for (R)-sotolon was -6.5° and for (S)-Sotolon was $+7.1^\circ$. The synthetic scheme is summarized in Figure 4.

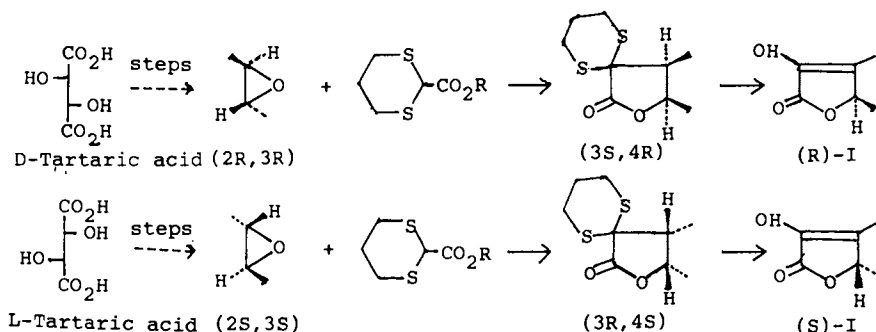


Figure 4. Stereospecific Synthesis of Sotolon Enantiomers

Both the enantiomers showed the same aroma character at the same concentration near the threshold value of (\pm) sotolon; moreover, there was no difference in the insect attractancy (house fly and cockroach) among the two enantiomers and the racemate of sotolon. Later, we(9) tried to analyze the aroma compound in fresh sugar cane juice and could not identify sotolon in the same fraction as that separated from cane molasses. These results suggest that sotolon was present in a racemic form prepared by mutual interaction of the constituents in sugar cane juice.

Formation of Sotolon

A burnt, sugary aroma is representative of some cooked foods. A number of sugary aroma compounds are listed below:

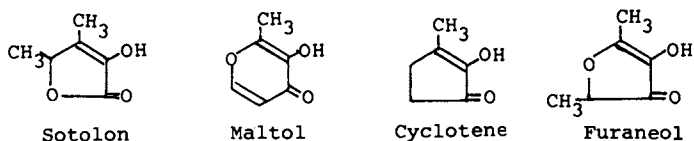


Figure 5. Sugary Aroma Compounds Having a Common Partial Structure

These have a partial common structure, i.e. enolized α -diketone and α -hydrogen to the enolizable carbonyl is substituted by a methyl group. For a characteristic sugary aroma, the presence of enol-hydrogen was essential, because the acetyl ester or methyl ether of sotolon showed no characteristic aroma. The extraordinarily low threshold value of sotolon may be due to its coexistence with hydrophilic and hydrophobic (alkyl-substituted lactone) partial structures in one molecule. The lower threshold value of the ethyl-substituted homolog (II) than that of sotolon may also be explained from the balance of these opposite physicochemical properties.

Apart from sotolon, the other compounds in Fig. 5 can be explained as the products of a Maillard reaction, and their carbon skeletons simply originate from the active Amadori intermediate; in other words, they still preserve the straight carbon chain structure of monosaccharides. In spite of being a simple C_6 lactone, sotolon has a branched carbon skeleton, which implies another formation process in the Maillard reaction. Sulser *et al.*(6) reported that ethyl sotolon (II) was prepared from threonine with sulfuric acid, and that 2-oxobutyric acid, a degradation product of threonine, was a better starting material to obtain II. This final reaction is a Claisen type of condensation, which would proceed more smoothly under alkaline conditions. As we(10) obtained II from 2-oxobutyric acid (see figure 6) with a high yield in the presence of potassium carbonate in ethanol, a mixed condensation of 2-oxobutyric and 2-oxopropanoic (pyruvic) acids was attempted under the same conditions, and a mixture of sotolon (22% yield) and II were obtained; however, the

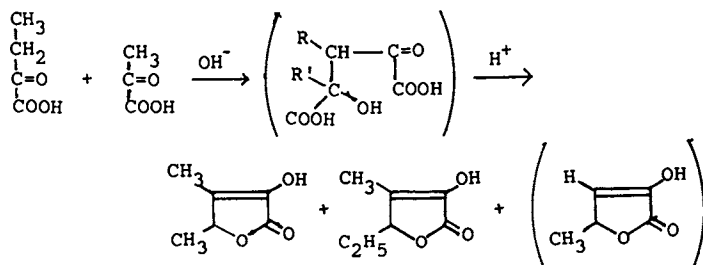


Figure 6. Preparation of Sotolon Homologues by a Mixed Condensation of 2-Oxoalkanoic Acid

self-condensation product of 2-oxopropanoic acid, desmethylsotolone, was not identified in the reaction mixture.

In the actual manufacturing process for sugar, there exist various Maillard reaction series, and pyruvate seemed to be a degradation product of carbohydrate. On the other hand, 2-oxobutyric acid could be derived from glutamic acid which is the main free amino acid in sugar cane juice as well as aspartic acid.(11) An equimolar amount of glutamic and pyruvic acids was dissolved in water, the pH was adjusted to 8 with potassium carbonate and the mixture boiled for 4 hours. Sotolon was identified in the ether extract of the reaction mixture by GC-MS the yield was below 0.1 %. From this data, the glutamate seemed to have been oxidized to α -keto glutarate in the presence of pyruvate, before it was condensed with the pyruvate and subsequent decarboxylation to yield sotolon. In practice, the characteristic aroma of brown lump sugar appears at the last manufacturing stage when the high-sugar-content liquid (Bx. 73) is heated at 135 °C; therefore, sotolon must be formed at this stage following the complex amino-carbonyl reaction just described. A speculative formation mechanism for sotolon and its homologues is shown in Fig. 7, in which an unstable keto acid oxidizes an amino acid to the α -keto acid, which then condenses with another keto acid to form the furanone structure. The last stage of the reaction is decarboxylation, which would be dependent on the pH and temperature conditions.

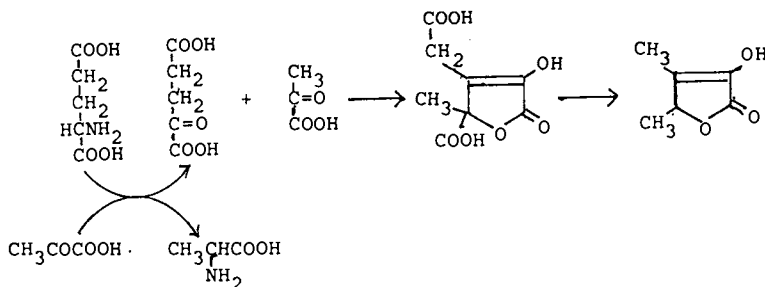


Figure 7. Formation of Sotolon during an Amino-Carbonyl Reaction

Flavoring Roles of Sotolon

When Japanese rice wine (sake) is kept under unsuitable conditions, it develops an off-flavor with a burnt or soy-sauce (shoyu)-like odor. Takahashi *et al.*(12) identified the main component as sotolon before our identification, and they claimed that the sotolon concentration was between 140-430 ppb in aged sake. This concentration is much higher than the threshold value of sotolon, and its aroma character would change from sugary to herbal or curry-like. The high sotolon content in aged sake could be a reason for the off-flavor defect. The formation of sotolon in aged

sake has also been proposed by the same author. Contrary to the heating conditions during cane sugar manufacture, the formation of this off-flavor proceeds at room temperature and under acidic conditions. They claimed that 2-oxobutyric acid and acetaldehyde reacted to produce sotolon in the presence of many organic acids. In this case, 2-oxobutyric acid was thought to have been derived from threonine (2.7 mM in Japanese sake). On the other hand, sotolon was found as the most important flavoring material in botrytized wine, (13), which is highly praised for its noble sweet flavor formed by infection with *Botrytis cinerea* fungus in ripe grapes. The subtle amount of flavoring material was separated by DEAE-sephadex column chromatography and identified by GC-MS. A quantitative analysis was also performed by mass fragmentography, and the concentration of sotolon in this wine was calculated to be 5-20 ppb. The same content levels were found in botrytized wines from Japan, France and Germany; however, in normal wines, the levels were lower than 1 ppb. By an organoleptic omission test on the aroma constituents in botrytized wine, it became clear that sotolon plays an important role in this special wine character. These results obtained from aged sake and botrytized wine show that sotolon can develop the off-flavor or be a desirable flavor substance depending on its concentration in the beverage. Sotolon has also been found in volatiles from roasted tobacco (14), and the addition of the condensate of the volatiles to cigarettes improved their aroma properties and decreased the offensive odor and taste (15). By combining the fractionation of the volatile condensate and an organoleptic evaluation, sotolon proved to be one of the active compounds with 4-hydroxy-2,5-dimethyl-3(2H)-furanone (furanol). Therefore, the formation of sotolon is possible under a wide range of conditions, from mild fermentation and preservation to more intensive roasting conditions. As described above sotolon shows its burnt sugary aroma at a ppm or ppb level; therefore, in order to prove the role of sotolon in the sugary aroma products, it became necessary to analyze sotolon quantitatively and qualitatively by applying the effective concentration for sensitive micro-analysis. By following this analytical system, we attempted to verify the presence of sotolon and establish its contribution to the aroma of raw cane sugar (not published). The aroma fraction was concentrated by silica gel column chromatography as already described and analyzed by GC-MS with a SIM (selected ion monitoring) system, in which the selected ion on the mass spectrometer was fixed and each GC peak scanned for this fragment ion. By fixing ions, the sensitivity of MS can be greatly increased to the pg order, and this makes it possible to identify quantitatively a very low concentration component such as sotolon. We selected the molecular ion (M^+ 128) for the SIM analysis and deduced the amount of sotolon in imported raw sugar (a raw material for the sugar refining process) to be between 0.1-0.01 ppb, which is enough to contribute the characteristic aroma to raw cane sugar.

Conclusion

With the development of various analytical instruments and techniques, it has become possible to identify minor but important aroma constituents. As the volatile aroma compounds generally have a

simple chemical structure, a FIC with very low concentrations in food was identified as a known compound after much tiresome and time-consuming work, even when using sophisticated methods of separation and identification. Our finding of sotolon as an FIC in raw cane sugar is one such example; moreover, through this study, we began to recognize that some aroma compounds change their odor character with concentration. If such a compound has a very low odor threshold value, it is important that an olfactory evaluation of the odor character in a diluted state near this threshold value be made. To investigate such an aroma compound, it became necessary to integrate all the results obtained by the various scientific approaches of organoleptic evaluation, syntheses of a target compound or its homologues, chemical or biochemical transformation in a model system and modern instrumental analyses. Flavor chemistry in the future will be of concern to an ever-widening scientific field, and will mature into a more sophisticated science.

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Chapter 6

Role of Oxidative Processes in the Formation and Stability of Fish Flavors

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The development of characterizing fish aromas and flavors involve both enzymic and nonenzymic oxidative reactions. Lipoxygenase-derived carbonyls and alcohols contribute the distinctive planty-green aroma notes to fresh fish that vary with species with regards to compounds and concentrations.

Additionally, key secondary oxidation products contribute distinctive aromas characteristic to certain fish species. In salmon, co-oxidation of polyunsaturated fatty acids of fish oils with salmon-specific carotenoid pigments leads to the formation of a characterizing cooked salmon flavor compound, and changes the ratio of carbonyl compounds formed compared to that for pure fish oil.

Nonenzymic autoxidation reactions which are predominant in highly unsaturated fish lipids, can be directed by the use of tocopherol-type antioxidants to manipulate oxidized flavors and thus influence the quality of fish aromas and flavors. Secondary oxidation of aldehydes to acids by peracids is responsible for the formation of short chain fatty acids (C₄ to C₈). These acids do not appear to contribute characterizing flavors and aromas in oxidizing fish lipids.

The development of both desirable and undesirable fishy flavors has long-been a concern to the seafood and fishery industry (1-6). Oxidative processes occurring through enzymic and nonenzymic mechanisms initiate hydroperoxide formation in fish lipid systems that are responsible for the formation of the short chain carbonyls and alcohols which exhibit distinct fish-like flavors and aromas. Because the generation of fresh fish aroma compounds involves some of the same polyunsaturated fatty acid precursors and oxidative pathways as autoxidation, it has been a tedious task to differentiate the mechanisms and aroma compounds

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contributing desirable fresh characteristics from those causing deteriorated fishy aromas and flavors.

Initiation processes for hydroperoxide siting on polyunsaturated fatty acids in fish occur by mechanisms encountered broadly in lipid oxidation, and include enzymic hydroperoxidation (7-11), singlet oxygen addition to double bonds (12-17), and classical Farmer-type H-abstraction and molecular oxygen addition to the pentadiene free radical (18-22). Considerable attention has been given to the identification of compounds derived from enzymically (23-27) as well as autoxidatively-produced (28-32) hydroperoxides of long chain n-3 fatty acids that are responsible for character-impact fish-like flavors and aromas. It now has been well documented that C₆, C₈ and C₉ aldehydes generated enzymically from n-3 fatty acids (23-27) are responsible for much of the planty, green-type aromas encountered in very fresh fish and seafoods.

Autoxidative reactions in fish or fish oils can also lead to green flavors (32), but these flavors are accompanied by elevated concentrations of compounds contributing general oxidized flavors as well as the 2,4,7-decatrienals and α -4-heptenal which are primarily responsible for burnt, oxidized-fishy or cod liver oil-like flavors.

Enzymically generated fish flavors and aromas

Lipoxygenase (10-11, 23-27), NADH-dependent microsomal oxidase (33-36), and myeloperoxidase (37-38) that are endogenous to fish tissue all apparently can participate in hydroperoxide formation on unsaturated fatty acids of fish lipids. Of these enzymes, however, only lipoxygenases provide positional hydroperoxide site specificity on the long-chain n-3 fatty acids, and such siting leads to specific compounds that initially cause characteristic fresh fish aromas. Differences in lipoxygenase enzymes in freshwater and saltwater species provide distinct profiles of volatile compounds that account for the species specific flavor and odor variations (23-27). Generally, both freshwater and saltwater fish contain substantial levels of hexanal and unsaturated C₈ alcohols which are responsible for the heavy, green character notes (23-27).

Nine-carbon compounds, such as 2-nonenal, 2,6-nonadienal and 3,6-nonadienal, occur mainly in freshwater species, and contribute to the fresh green melon-like quality (23,26,27). Pacific salmon entering freshwater from saltwater enroute to spawning produce the nine-carbon compounds, but only the C₆ and C₈ carbonyls and alcohols are found in saltwater-dwelling salmon (39). Such a transition to the production of the nine-carbon compounds along with the C₆ and C₈ compounds has been suggested to be related to the induction of physiological processes that increase slime formation, and possibly provide improved osmoregulation by salmon in freshwater environments. The resulting variations in lipid-derived compounds during the life cycle of salmon, however, influence the aroma and flavor of fish harvested at various times.

A 12-lipoxygenase found in trout gill extracts (10-11) is responsible for the siting of the n-9 hydroperoxide on

eicosapentanoic acid (EPA) and docosahexanoic acid (DHA). This site specific hydroperoxide formation in conjunction with lyase activity results in the formation of 3,6-nonadienal, 2,6-nonadienal and 3,6-nonadien-1-ol which provides the fresh, green-cucumber quality of certain very fresh fish, such as the lake whitefish (*Coregonus clupeaformis*; 23,27). The presence of the 15- and 12-lipoxygenases in fish provides specificity for the siting of n-6 and n-10 hydroperoxides on n-3 fatty acids, respectively. These enzymes are postulated to account for the biogenesis of the unsaturated C₆ and C₈ carbonyls and alcohols (39). These aroma compounds contribute a heavy, plant-like character to fresh fish, and their formation parallels similar reactions on shorter-chain, unsaturated fatty acids in fruits and vegetables (40-42). The existence of a 5-lipoxygenase in fish has been suggested (39) in relation to the formation of short chain oxoacids, but evidence for this enzyme is circumstantial at the present time.

The myeloperoxidase/halide system found in fish has been shown to produce elevated levels of superoxide anions and hydrogen peroxide which in turn randomly peroxidizes the olefin bonds of fish lipids (37-38). The NADH-dependent oxidase system, which was isolated as a membrane fraction from fish skeletal muscle, requires ADP and iron ions to initiate hydroperoxidation, but its mechanism of activity is still unclear (33-36).

Singlet oxygen-generated hydroperoxides

Singlet oxygen-mediated hydroperoxidation reactions are random processes of initiation because of the non-selective interactions of singlet oxygen for any olefin in unsaturated systems. The increased energy state of molecular oxygen provided by spin inversion to form the higher energy singlet state allows for direct olefinic interaction with singlet oxygen (16, 17). The mechanism of excitation involves photosensitizers that allow spin conversion to occur, and natural photosensitizers in foods include porphyrin ring structures, such as heme and chlorophyll, which possess conjugated sites of unsaturation for electron delocalization and transfer (16, 17, 43).

The presence of photosensitizers in fish oils depends on the method of processing and the source of fish used for rendering. Some species of fish, such as menhaden, which are primarily phytoplankton feeders, can easily contribute residual chlorophyll from their stomach contents when oils are rendered. Fish liver oils have a likelihood of containing residual heme compounds, and thus both oils would be generally more vulnerable to singlet oxygen autoxidation than oils recovered from dressed fish.

Although singlet oxygen hydroperoxidation is a non-specific initiation reaction, it provides an opportunity for siting hydroperoxides at otherwise unfavored positions on polyunsaturated fatty acids (44-46). In oxidizing cod liver and menhaden oils a compound that exhibits an odor reminiscent of vegetables and cooked celery which may contribute to green notes of oxidizing fish oils has been tentatively identified as 2-hydroxy-3-pentenalactone (32). The formation of

2-hydroxy-3-pentenalactone likely would require the siting of a hydroperoxide at the 5-position of DHA followed by reactions leading to the cyclization of the unsaturated 5-oxo-3-pentenoic acid. A siting of this hydroperoxide at the 5-position on DHA would not be favored in pentadienyl free radical autoxidation (44-46), but could be readily formed by a singlet oxygen mechanism.

Farmer-type autoxidation-derived fish flavors

The now classic Farmer-type hydrogen-abstraction initiation of free radical autoxidation accounts for a large portion of the nonenzymic oxidations of n-3 fatty acids (45). Because fish lipids contain substantial concentrations of EPA and DHA (47-48), they provide many allowed sites (18, 22, 45, 46, 49) of hydroperoxide formations, and thus can account for a large array of decomposition products. Oxidizing model systems of unsaturated methyl esters of fatty acids yielded monohydroperoxides, but also produce dihydroperoxides that are formed by cyclization of intermediate hydroperoxy radicals when suitable H-donating antioxidants are not present to quench the free radical reaction (45, 50, 51). Decomposition of monohydroperoxides of fatty acids in model systems yields a very different profile of lower molecular weight products than observed for similar decompositions of dihydroperoxides of the same fatty acids (45, 46).

Only a limited number of volatile products generated from n-3 fatty acids provide characterizing green, fishy or burnt flavors of oxidizing fish and fish oils. Allowed-site monohydroperoxide formations (18, 22, 45, 46, 49) and decompositions leading to some of the characterizing aldehydes via Farmer-type reactions in fish oils are summarized in Figure 1. As noted earlier, some aldehydes responsible for the green flavors and aromas in autoxidizing fish oils are common with those solely generated enzymically in freshly killed fish (23). Differences in concentrations of these and other compounds account for the overall aroma or flavor quality of fishery products exclusive of those contributed by microbial spoilage.

Of the aldehydes generated from the classic Farmer autoxidation mechanism, the 2,4,7-decatrinal isomers have been identified as being highly contributory to burnt/fishy or cod liver oil-like flavors (28, 32). Typically, both isomers exhibit the burnt/fishy flavor character, but the *t,t,c*-isomer can also contribute a green-fishy character to oxidizing fish oils at low concentrations (28, 32).

Directing effects of tocopherol-like antioxidants

Antioxidant systems for fish oils frequently contain alpha-tocopherol, but these systems and those based on phenolic antioxidants do not provide adequate protection against the formation of fishy flavors in fish oils. Swoboda and Peers (52-53) investigated a metallic/fishy flavor that developed only

in fractions of butteroil containing alpha-tocopherol and copper. These workers reported that 1,5-octadien-3-one from the oxidation of long-chain polyunsaturated n-3 fatty acids was responsible for metallic notes, but the fishiness appeared to be contributed by the 2,4,7-decatrienals or a combination of these compounds. This relationship has been explored further in our laboratory through mechanistic studies employing alpha-tocopherol and Trolox C which is a synthetic tocopherol-type antioxidant (Figure 2).

Since alpha-tocopherol was an essential component of the Swoboda and Peers system (52) which yielded metallic/fishy flavors, its structural role in the directing of the flavor was of interest. Either alpha-tocopherol (670 ppm) or Trolox C (1000 ppm) were added to steam deodorized menhaden oils (2 h at 130°C, 4 mm Hg; 32) and allowed to oxidize while exposed to air and protected from the light at 65°C. As observed in previous studies (54-56), the high level of alpha-tocopherol exhibited a prooxidant effect whereas Trolox C possessed a distinct antioxidant effect in oxidizing menhaden oils (57).

Both alpha-tocopherol and Trolox C are capable of donating a hydrogen atom radical from the hydroxyl group of the chroman ring (Figure 3) to quench free radicals in oxidizing lipid systems (57-58). Differences in the stability of the chroman free radical yielded by these two compounds account for the different antioxidant properties exhibited. Although alpha-tocopherol readily donates its hydrogen atom in the initial stage of free radical quenching, the resulting chroman free radical is an effective competitor for abstraction of hydrogen atoms from unsaturated lipids or other hydroperoxides in oxidizing systems (58). Consequently, this reverse process allows for the promotion of oxidation by forming increased levels of free radicals. On the other hand, Trolox C, having a strong electron withdrawing carboxyl group substituted for the alkyl chain of alpha-tocopherol (Figure 2), more readily undergoes the two step oxidation process to form the corresponding oxidized lactone or quinone (Figure 3; 57). Formation of the quinone not only prevents the reversible H-abstraction from occurring, but it also allows for the quenching of an additional free radical in the process (Figure 3).

Headspace volatiles from the experimentally oxidized fish oils were analyzed quantitatively by the method of Olfasdottir et al. (59), and revealed that tocopherol-type antioxidants in samples increased the formation of 1,5-octadien-3-ol and 1,5-octadien-3-one compared to a control (Figure 4). Trolox C had a more pronounced effect than alpha-tocopherol. A proposed mechanism for the enhanced formation of the two C₈ compounds is illustrated in Figure 5, and involves an alignment of the pi-bond clouds of the oxidized quinone with the n-3 end of the fatty acid fragment. This alignment provides the desired geometry for the transfer of the hydroxyl group to the 3-position of the 8-carbon fragment. Because 1,5-octadien-3-ol is produced more abundantly than the corresponding ketone, it appears that direct hydroxylation is favored over an oxygen transfer to form 1,5-octadien-3-one. As indicated in Figure 5, a possibility

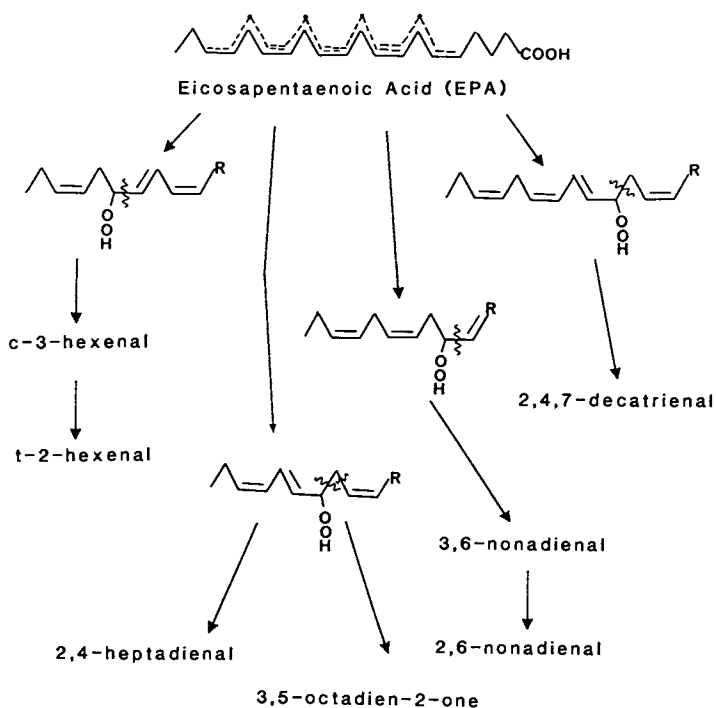


Figure 1. Carbonyls associated with characterizing fish flavors produced from the autoxidation of eicosapentaenoic acid.

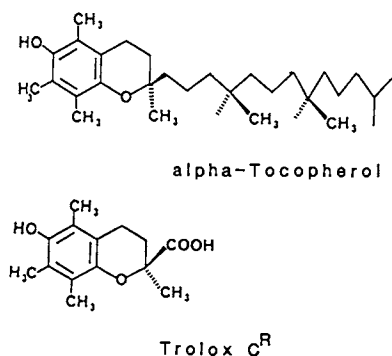


Figure 2. Structures of tocopherol-type antioxidants evaluated in oxidizing fish oils.

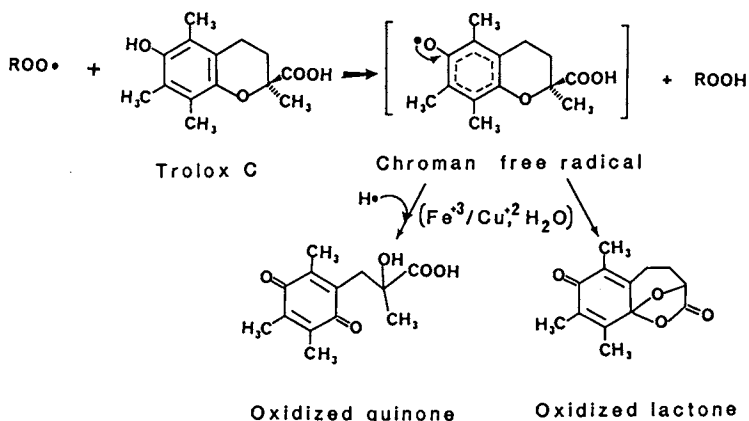


Figure 3. Antioxidant mechanism of Trolox C.

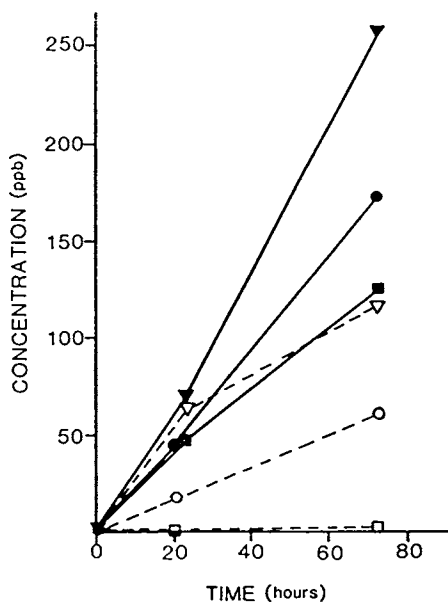


Figure 4. Formation of 1,5-octadien-3-ol and 1,5-octadien-3-one in the presence of tocopherol-type antioxidants in menhaden oil under accelerated conditions. [$\blacktriangledown, \blacktriangledown$ Trolox C; \bullet, \circ alpha-tocopherol; \blacksquare, \square control; (---), 1,5-octadien-3-one; (—), 1,5-octadien-3-ol].

exists for the formation of the ketone from the alcohol through an oxidation reaction.

Theoretically, the alignment of the electron clouds of the molecules could facilitate both the hydroxyl group siting and the cleavage of the fatty acid backbone to provide the C₈ fragment, or alternatively the hydroxylation could occur after C₈ fragment has been released by an unrelated event. Since Trolox C is more readily oxidized to the quinone structure than alpha-tocopherol (57), the large concentration of unsaturated C₈ compounds produced in the presence of Trolox C supports a hypothesis that the directing action is more likely dependent on the formation of the oxidized chroman ring rather than the alignment character that may be provided by the alkyl chain.

When 20 ppm of Cu⁺⁺ (as cupric palmitate) was added to deodorized cod liver oil, similar effects of alpha-tocopherol and Trolox C on the production of 1,5-octadien-3-ol and 1,5-octadien-3-one were observed except that even higher concentrations of the C₈ compounds resulted (Table I). This observation may be explained by the catalytic effect of metal ions in the formation of the oxidized quinone (57). Incorporation of Trolox C and copper caused the production of about four times the concentration of the unsaturated C₈ compounds compared to the sample containing alpha-tocopherol and copper. The sample containing Trolox C exhibited a strong metallic, vinyl ketone-like aroma quality (Table I) which probably was of the type described by Swoboda and Peers (52-53). Alpha-tocopherol and copper in fish oils, however, also allowed the development of high concentrations of the 2,4,7-decatrienals which resulted in an obscuring of the metallic note and the expression of strong cod liver oil-like aroma and flavor.

The ready donation of H-radicals from the hydroxyl group on the chroman ring quenches peroxy radicals formed on partially oxidized polyunsaturated fatty acids, and thus prevents intramolecular cyclizations that can lead to the formation of dihydroperoxides (22, 45, 50, 51). Thus, monohydroperoxide degradations lead to the fishy long-chain unsaturated aldehydes while diperoxide decompositions form shorter chain compounds (45-46) that contribute only to the general oxidized, painty flavor of oxidizing fish oils.

The H-donating character of the tocopherol-type compounds also causes a preferential formation of cis-trans rather than trans-trans monohydroperoxides that provide the direct precursors of the 2,4,7-decatrienals causing burnt/fishy flavors. A stepwise mechanism for the formation of t,t-hydroperoxide compared to t,c-hydroperoxide has been proposed by Porter et al. (51, 60). These researchers suggested that once the peroxy radical formed on the molecule, carbon-carbon bonds could rotate to establish the initial trans configuration (step 1; Figure 6). After bond rotation, it was proposed that the peroxy free radical could translocate along the carbon backbone (step 2, Figure 6) resulting in alternate siting at an allowed pentadienyl position on the

Table I. Odor and concentrations of selected compounds forming in oxidizing cod liver oils incorporating selected antioxidants and held at 65°C for 16.5 h

Sample description	1,5-Cg-3-ol	1,5-Cg-3-one	<u>t</u> , <u>c</u> , <u>c</u> -2,4,7- <u>t</u> , <u>t</u> , <u>c</u> -2,4,7-decatrifenal	Odor description	
	-----concentration (ppb)-----				
Control	65	tr ^a	320	400	green; fishy
670 ppm alpha-tocopherol + 20 ppm Cu ⁺⁺	669	75	2710	1370	very cod liver oil-like; straw-like
1000 ppm Trolox C + 20 ppm Cu ⁺⁺	3480	264	1200	390	metallic; vinyl ketone-like

^aTrace.

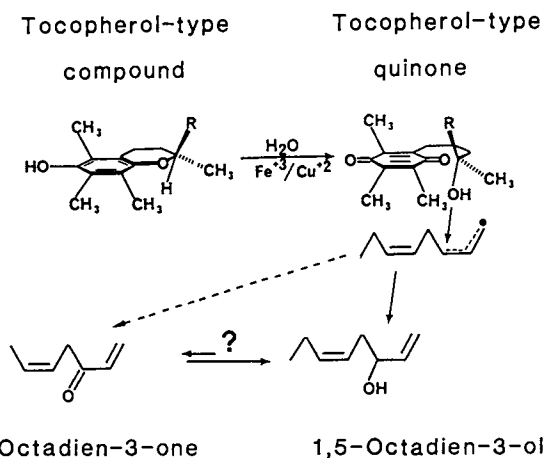


Figure 5. Proposed mechanism for the directed oxidation by tocopherol-type antioxidants for *n*-3 fatty acid fragments leading to the formation of C_8 compounds.

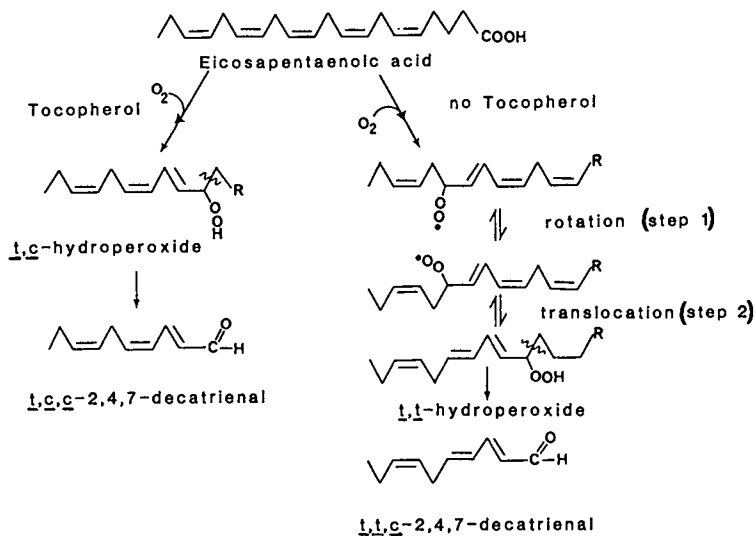


Figure 6. Formation of isomeric hydroperoxides of polyunsaturated fatty acids.

molecule. In the presence of strong H-donating antioxidants, the immediate quenching of peroxy radical would prevent the rotation of the carbon-carbon bond as well as the translocation of the peroxy radical on the unsaturated fatty acid, and thus minimize the formation of trans-trans hydroperoxide isomer formation (Figure 6; 41, 50, 60, 61).

The directed formation of the t,c,c-decatrinal isomer compared to the t,t,c-isomer in fish oil is illustrated by the ratio comparisons shown in Table II. In samples of cod liver oil containing either alpha-tocopherol plus copper or Trolox C plus

Table II. Ratios of selected C₈ and C₁₀ carbonyls produced from oxidized cod liver oils containing tocopherol-like antioxidants

Sample description	Ratio of volatile compounds			
	C ₈	<u>t,c,c</u> -C ₁₀	C ₈ -ol	C ₈ -ol
	C ₁₀	<u>t,t,c</u> -C ₁₀	<u>t,c,c</u> -C ₁₀	<u>t,t,c</u> -C ₁₀
Control	0.09	0.80	0.20	0.16
670 ppm alpha-tocopherol + 20 ppm Cu ⁺⁺	0.18	1.98	0.24	0.49
1000 ppm Trolox C + 20 ppm Cu ⁺⁺	2.35	3.08	2.94	8.92

copper, at least twice the amount of the t,c,c-2,4,7-decatrinal isomer was produced as compared to the control. Since Trolox C exhibits high non-reversible H-donating antioxidant properties, its ability to more effectively direct toward the t,c,c-isomer is seen by an elevated isomer ratio of 3.08 in the presence of Trolox C (Table II). As noted earlier, low concentrations of the t,c,c-isomer of 2,4,7-decatrinal exhibit a more pronounced fishy/burnt flavor quality in oxidizing fish oils while the t,t,c-isomer causes more green-fishy, burnt character notes (28). Although substantial concentrations of the C₈ unsaturated compounds were produced in the fish oils containing Trolox C or alpha-tocopherol (Table I), the production of 2,4,7-decatrinals provided the underlying unpleasant, characterizing fish flavor notes.

Since both the C₈ and C₁₀ unsaturated aldehydes are derived from the n-3 end of polyunsaturated fatty acids, the aroma and flavor profile of lesser oxidized fish oils would be expected to reflect the influences provided by the antioxidant system. Since Trolox C favors formation of C₈ unsaturated compounds compared to the unsaturated C₁₀ compounds during the oxidation of fish oils (C₈:C₁₀ ratio = 2.35; Table II), it is likely

that less fishy aromas will result from its use, but metallic notes from the C₈ compounds may likewise cause problems with flavors. However, it may be possible to mask metallic flavors more readily than the fishiness caused by the 2,4,7-decatrienals.

Co-oxidation of carotenoids and fish lipids

Recent studies on salmon flavors revealed that a single compound appears to be responsible for the characterizing cooked salmon flavor (39). The cooked salmon flavor compound was found to have an extremely low threshold, and was initially detected only by odor assessment of a fraction eluting at I_R of 9.6-9.7 on a Carbowax 20M packed column when headspace volatiles were analyzed from canned salmon meat. Accelerated oxidation of salmon oil did not yield salmon-like aromas before the development of fishy oxidized aromas. However, when salmon oil was coated onto Celite supports, and allowed to oxidize at room temperature, a distinct salmon-loaf-like aroma developed within 24 h after initiation of oxidation. A variety of supports were evaluated in model systems with salmon oil for their ability to produce the salmon aroma compound. Odor assessments of the oxidizing systems Table II indicated that a range of odors developed from salmon-loaf-like to oxidized fishy aromas, and only the Celite system provided the aroma.

The interaction of the carotenoid and the fatty acid fractions on Celite were both necessary for the odor development to occur. Studies designed to confirm an interaction of the carotenoid and fatty acid fractions in the development of salmon flavors showed that when carotenoid fractions from salmon oils were separated from the acylglycerol fraction by column chromatography, neither yielded a salmon-like aroma during oxidation (Table III). However, when the carotenoids and acylglycerols were recombined, the salmon aroma developed. Combinations of alternate sources of fish acylglycerols along with crayfish carotenoids revealed that the necessary component for salmon flavor development was the presence of carotenoids specifically derived from salmon oil (Table III). Such results strongly suggest that the compound is derived by co-oxidation of fish acylglycerols with salmon carotenoids, and that the precursor is located in the carotenoid fraction.

The GC-collected salmon flavor compound was analyzed by mass spectrometry and found to possess a molecular weight of 138 and mass fragments reminiscent of an alkyl furanoid-type structure. A compound fitting this type of molecular configuration was suggested as being ethyl-(3-methyl furyl)-ketone which would accommodate the analytical evidence (39). Formation of such a compound from salmon carotenoids would likely require an allene grouping in a carotenoid which could lead to the formation of a ring compound.

In addition to the directed oxidation process responsible for the characteristic salmon flavor compound, carotenoids also were observed to influence oxidation of polyunsaturated fatty acids in salmon oil systems (39). Typically, lipid oxidation of oils high in n-3 fatty acids produce isomeric 2,4-heptadienals as the major

Table III. Carotenoid and fatty acid contributions to cooked salmon flavor^a

Oil/Celite System	Overall Aroma Quality
<u>From Salmon Oil</u>	
Carotenoid	unsaturated hydrocarbon
Acylglycerol	rancid fish
Carotenoid + Acylglycerol	cooked salmon loaf
<u>From Other Sources</u>	
Salmon carotenoid + Menhaden oil	cooked salmon loaf
Crayfish carotenoid + Salmon acylglycerol	no salmon aroma
Salmon acylglycerol + Menhaden oil	rancid fish

^aFrom Josephson, 1987 (39).

autoxidation products, and the isomeric 3,5-octadien-2-ones are present as less abundant breakdown products (30, 31, 61). Studies employing salmon oil containing carotenoids as well as fractionated salmon oil devoid of carotenoids showed that the ratio of the 2,4-heptadienal isomers and the 3,5-octadien-2-one isomers were very different in the two systems. Although all of the isomers of both of these lipid oxidation products are derived from the n-7 hydroperoxide of n-3 fatty acids, a very distinct difference in the breakdown of this hydroperoxide occurred in the presence and absence of the carotenoid fraction. These particular oxidation products are not considered to provide characterizing fish flavors, but their varying contributions would likely be notable in the overall quality of fish aromas and flavors.

Role of oxidatively-produced saturated fatty acids in fish flavors

We originally believed that the short chain saturated fatty acids in oxidizing fish lipids contributed to burnt/fishy flavors. Saturated fatty acid concentrations (C₄ - C₉) measured by volatile headspace analysis (32, 59) reached levels as high as 3 ppm in highly oxidized fish oils (32). Flavor thresholds for these short n-chain fatty acids in oil systems in the literature (>.66 ppm, 63) indicate that they could contribute notes to oxidizing fish oils. However, studies designed to document the role of short chain acids as flavor compounds detracting from the flavor quality of fish oils did not confirm earlier beliefs.

Short chain fatty acids are formed by peracid oxidations of autoxidatively-derived n-alkanals (63), and the incorporation of the peracid inhibitor, dilauryl thiodipropionate (63), into deodorized fish oils was investigated as a means of preventing the formation of fishy/burnt flavors. Although results of these studies showed substantial reduction of these acids to concentrations well below threshold (<10 ppb), burnt/fishy flavors

were still pronounced and unaffected. When concentrations up to 20 ppm of either butyric, pentanoic, hexanoic, heptanoic and octanoic acids were added to deodorized fish oils and bland vegetable oils, only butyric acid gave a weak cheese-like, buttery aroma (32). Therefore, it was concluded that short n-chain fatty acids found in oxidizing fish oils were of insignificant concentrations to contribute characterizing burnt/fishy flavors and aromas.

Summary

Although it is well established that flavors generated from oxidative processes in fish and fishery products are difficult to control, some mechanisms for altering or directing predominant oxidative pathways to alternate flavors and aromas exist. We have only begun to unravel these complex relationships in fish flavor systems, but the results provide encouragement toward the goal of one day controlling the distinctly fishy flavors produced from the oxidation of long-chain polyunsaturated fatty acids.

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Chapter 7

Kinetics of Formation of Alkylpyrazines

Effect of Type of Amino Acid and Type of Sugar

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Pyrazines are heterocyclic, nitrogen-containing compounds important to the flavor of many foods. Prior studies relating to the effects of type of amino acid and type of sugar on the formation of pyrazines have yielded contradictory results. This study investigates the effects of type of amino acid and type of sugar on the kinetics and distribution pattern of pyrazines formed. The amino acids, lysine and asparagine, and the sugars, glucose, fructose and ribose were chosen for this study. One-tenth molar sugar/amino acid solutions buffered at pH 9.0 were heat-processed. Samples were analyzed using a headspace concentration capillary gas chromatographic technique with nitrogen-selective detection. Rate of pyrazine formation fit pseudo zero order reaction kinetics. Effects of amino acid and sugar types on activation energies, yields and relative distributions of pyrazines are discussed.

Pyrazines are heterocyclic, nitrogen-containing compounds important to the flavor of many foods. Alkylpyrazines have often been found in heated foods and have been characterized as having roasted, toasted, nutty flavor notes. Some excellent reviews have previously detailed the presence of pyrazines in a great variety of foods. Maga and Sizer (1, 2) published the first comprehensive reviews on pyrazines in foods. They reviewed the occurrences of numerous pyrazines in a wide variety of foods, pyrazine isolation, concentration, separation and identification techniques, pyrazine flavor properties, pyrazine mechanisms of formation and pyrazine synthesis techniques. Since then several others (3 - 10) have reviewed progress in pyrazine research. Understanding both the effects of various parameters on the kinetics of the formation of pyrazines and the mechanism of formation may allow for the

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optimization of pyrazine production in both foods and in reaction flavors.

Alkylpyrazines are most commonly found in roasted and toasted foods and are believed to form as a result of the Maillard browning reaction (11). Several researchers have proposed mechanisms for alkylpyrazine formation in various carbohydrate/amine systems (12 - 21). These pathways generally involve the formation of aminocarbonyl fragments which condense, yielding dihydropyrazines or hydroxy dihydropyrazines. These in turn yield pyrazines through oxidation (17) or dehydration reactions (18, 19). Aminocarbonyl fragments result through various Maillard reaction pathways. Some researchers postulate mechanisms in which free ammonia formed as a result of amino acid decomposition reacts with sugars and sugar fragments yielding alkylpyrazines (15, 16). Others have proposed mechanisms by which sugars and amino acids condense through the generalized Hodge Maillard reaction scheme (11, 12, 13) and Strecker degradation of amino acids with dicarbonyl fragments (17). Shibamoto and Bernhard (18) proposed the most detailed scheme of pyrazine formation pathways in sugar-ammonia model systems, involving α -aminocarbonyl intermediates which condense to form alkylpyrazines.

Various researchers have investigated factors affecting both yields of individual pyrazines and their distributions, including source of nitrogen and source of carbon. Often these studies have yielded seemingly contradictory results. The effect of source of nitrogen on pyrazine formation was initially investigated by Newell et al. (13). They reacted various amino acids with glucose and demonstrated that qualitatively the same volatile pyrazine compounds were produced regardless of the amino acid employed as the nitrogen source. Van Praag et al. (15) obtained similar results in reacting glycine, leucine, isoleucine, valine and alanine with D-fructose. Koehler et al. (16) observed formation of a similar series of pyrazines when reacting glucose with asparagine, glutamine, glutamic acid and aspartic acid. Mostly alkylated pyrazines were found, with only traces of unsubstituted pyrazine. However, in reacting ammonium chloride with glucose, unsubstituted pyrazine was the main pyrazine observed with only traces of alkylated pyrazines being found. Also, different pyrazine product distributions and total pyrazine concentrations were obtained for the different amino acids. Wang and Odell (14) reacted glycerol with alanine and glycine. Similar series of pyrazines resulted, however total yields and distributions were different. Most recently, Wong and Bernhard (20) demonstrated that the distribution and yield of pyrazines formed in the reactions of glucose with ammonium hydroxide, ammonium formate, ammonium acetate, glycine and monosodium glutamate depended strongly on the nature of nitrogen source.

The effect of source of carbohydrate on pyrazine formation was first investigated by van Praag et al. (15). The same series of pyrazine compounds was isolated from a reaction mixture of fructose or glucose with ammonia. Koehler et al. (16) found that the carbohydrate source affected the total yield of pyrazines formed. In reacting 10 mmoles each of a sugar with ammonium chloride, total yield of pyrazine was 59 μ moles with glucose and

195 μ moles with fructose. Koehler and Odell (22) monitored production of methylpyrazine and dimethylpyrazine in reacting asparagine with glucose, fructose, sucrose and arabinose. They found the carbon source to affect both total yield and relative distribution. In a more comprehensive study, Shibamoto and Bernhard (18) reacted ammonia with several sugars. Reaction systems consisted of a solution of 8M ammonium hydroxide and 1M carbohydrate, heated at 100°C for 2 h. They concluded that pentoses give greater total yields of pyrazines than hexoses, and yields from mannose, glucose and fructose are about equal with galactose giving slightly lower yields. Distribution patterns were essentially similar for the pentoses and hexoses, except aldoses gave more unsubstituted pyrazine than the ketose, fructose. They concluded that epimers, diastereomers and enantiomers gave identical pyrazine pattern distributions, contradicting the work of Koehler and Odell (22).

Our study was conducted to determine the effects of type of amino acid and type of sugar on both the kinetics and the distribution pattern of alkylpyrazines formed. For investigating the effect of type of amino acid, this work focused on two amino acids, lysine and asparagine. Lysine was chosen because it contains two amino groups available for reaction. Asparagine was chosen because Koehler and Odell (22) reported that yields of pyrazines were greatest when this amino acid was reacted with glucose, versus alanine, glycine or lysine. For investigating the effect of sugar, glucose, fructose and ribose were chosen for this study. Glucose and fructose were chosen to provide two common hexoses in foods, one an aldose and one a ketose. The pentose ribose was chosen because of its reportedly high degree of reactivity in meat reaction flavor systems (Dwidedi, 23).

The present investigation used a headspace concentration capillary gas chromatographic technique with nitrogen-phosphorus detection. Advantages of this technique are that the procedure was rapid (about 30 minutes), potential for artifact formation is minimized and sample requirements are small (15 ml).

Experimental Section

The amino acids, sugars, borate salts and solvents were all reagent grade, obtained from commercial sources. The amino acid-sugar combinations investigated in the present study included asparagine-fructose, asparagine-glucose, lysine-fructose, lysine-glucose and lysine-ribose at concentrations of 0.1M for both amino acid and sugar, in a pH 9.0 0.1M borate buffer (24). Ten ml of each solution were heated in Teflon-capped 25 mm (o.d.) x 150 mm Pyrex test tubes in a water bath at 75, 85, and 95°C for up to 24 h. Samples were taken at 7 to 8 time intervals. Eighteen to 20 total samples per temperature were analyzed.

Although the amino acid/sugar solutions were buffered in an effort to maintain pH, a drop in pH was encountered with increasing reaction times. Therefore, after heat treatment, each sample was adjusted to pH 9.0 with 0.1N NaOH. One ml of a solution containing 2-methoxypyrazine in distilled water (2 ppm) was added as an internal standard. Final sample volume was 15 ml. Pyrazines were then isolated, separated and quantified using an

automated headspace concentration sampler (Hewlett Packard 7675A Purge and Trap) coupled to a Hewlett Packard 5880A gas chromatograph with nitrogen-phosphorus detection. The 15 ml sample was attached to the purge and trap sampler and purged for 10 min with hydrogen at a flow rate of 90 ml/min. Volatiles were adsorbed on a 4" by 1/4" o.d. stainless steel precolumn packed with Tenax (Hewlett Packard Co., Avondale, PA). At the end of the purge period, the sample was removed from the sampler before manually switching to the desorb cycle. Elution of the concentrated organic volatiles from the Tenax precolumn onto the GC column was accomplished by heating the precolumn to 180°C with a hydrogen flow of 90 ml/min. The combined flow of hydrogen and volatiles was split 50:1 and passed onto the GC column, a 25 m by 0.32 mm (i.d.) DB 225 fused silica capillary column (J & W Scientific Inc., Rancho Cordova, CA). The column head pressure was maintained at 15 psig which provided a linear velocity of 45 cm/sec and a flow rate of 4 ml/min. The volatiles were desorbed for 3 min from the Tenax column onto the chromatographic column. During this period, the volatiles were cold-trapped at the head of the chromatographic column by immersing a 20 cm loop of the column in a 2 3/8" i.d. x 4 1/2" Dewar flask containing liquid nitrogen. The oven temperature was isothermal at 50°C for the run, with a post value of 200°C for 1.5 min. The injection port and NPD temperatures were 225°C and 280°C, respectively. Figure 1 gives a typical chromatogram obtained by this procedure.

Quantification of the pyrazines was accomplished using an internal standard method. 2-methoxypyrazine was chosen as internal standard due to its similar physical properties (MW, solubility) to the pyrazines of interest and because methoxypyrazines have never been reported to form as a result of the Maillard reaction. The amounts of each pyrazine present in the sample were determined by the relationship:

$$\text{Amt. C} = \frac{\text{Amt. ISTD}}{\text{RF}} \times \frac{\text{AC C}}{\text{AC ISTD}} \quad (1)$$

where RF = response factor of the compound, C,
relative to the internal standard,
ISTD

AC = area count

Amt. = amount in µg/ml

Response factors were empirically determined by adding known amounts of each compound to 15 ml of pH 9.0 borate buffer, followed by a purge of the sample under usual conditions of analysis.

Pyrazine peak identification was initially accomplished by cochromatography with standards, (Pyrazine Specialties, Atlanta, GA) then further confirmed by gas chromatography/mass spectrometry using the following sample, equipment and operating conditions. A 10 ml sample of 0.1M glucose-lysine heated at 95°C for 6 h was analyzed. Following pH adjustment to 9.0 with 0.1N NaOH, the volume was brought up to 15 ml. The sample was analyzed using the Hewlett Packard 7675A Purge & Trap sampler interfaced with a Carlo

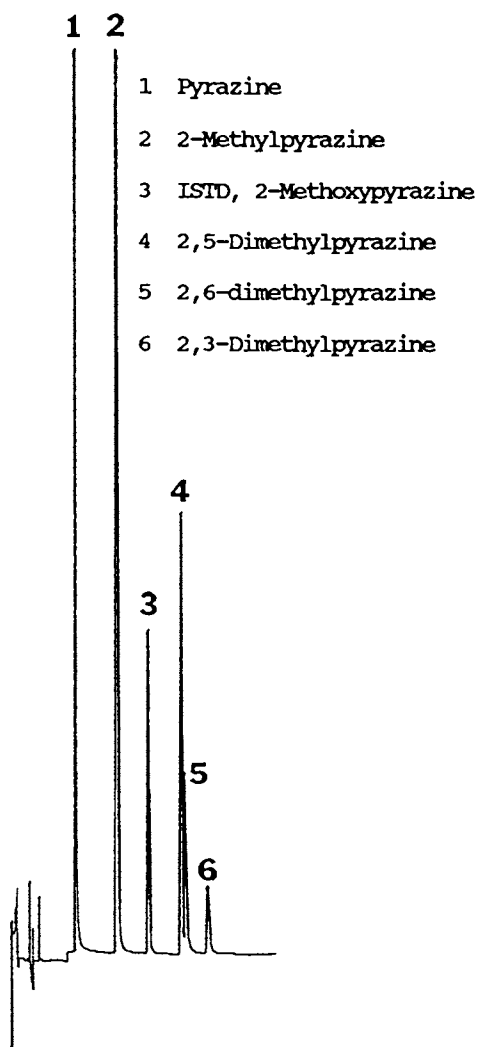


Figure 1. Chromatogram of pyrazines detected under standard conditions of analysis

Erba gas chromatograph connected to a Kratos MS 25 mass spectrometer. Helium was used as carrier at a pressure of 90 kPa. The same column was used for routine pyrazine analysis, a 25 m x 0.32 mm i.d. DB 225 fused silica capillary column. The run was isothermal at 50°C with a post value of 200°C for 2 min. Spectra were recorded at 70 electron volts. The spectra were compared to published spectra (25) as well as those obtained from reference standards.

The kinetics of the formation of pyrazines were determined using the basic equation for the rate of change of A with time:

$$\frac{dA}{d\theta} = kA^n \quad (2)$$

where A = concentration of pyrazine (ppm)
 θ = time (h)
 k = rate constant
 n = reaction order

Integrating this equation between A_0 , the concentration of A at time zero, and A, the concentration of A at time θ , yields

$$A = A_0 + k\theta \quad (3)$$

for a zero order reaction. This implies that the rate of formation of A is constant with time and independent of the concentration of reactants. For a first order reaction this yields the relationship:

$$\ln A = \ln A_0 + k\theta \quad (4)$$

In this case, the rate for formation of A is dependent on the concentration of reactants remaining. Reactions in foods have been found to follow pseudo zero or first order kinetics (26). One is generally safer when discussing reaction orders in foods in using the term "pseudo", due to the complexity of the system. Pseudo reaction orders in foods are generally assigned because a high correlation (r^2) for a mathematical relationship between formation of product and time exists.

The formation of pyrazines appears to better fit a pseudo zero order reaction rather than first order reaction. Plotting concentrations of pyrazines formed versus time of reaction gave the better fit of the line, usually with a coefficient of determination (r^2) of greater than 0.95. For a pseudo first order reaction, a curve rather than a line was obtained. General least squares analysis of the data was used to compute rate constants (27). Two zero points were used for each regression. Duplicate samples were tested at the early sampling times vs. triplicate samples at later times, as variations in concentration among replicates increased with increased reaction time. Each data point collected was treated separately in the regression analyses.

Activation energies for the formation of pyrazines were calculated using the Arrhenius relationship, which relates the rate constant, k, to temperature:

$$k = k_0 - E_a/RT \quad (5)$$

where k_0 = a pre-exponential (absolute) rate constant
 E_a = activation energy in kcal/mole
 R = gas constant, 1.986 cal/mole $^{\circ}K$
 T = temperature in $^{\circ}K$

Results and Discussion

Table I lists the regression data for the formation of pyrazines with time for the different sugar-amino acid combinations. An increase in rates of formation occurred with an increase in temperature. The effect of temperature on the formation of pyrazine in the lysine-glucose system is graphically depicted in Figure 2. Formation of pyrazines best fit a pseudo zero order reaction, with coefficients of determination usually greater than 0.90. This suggests that the rate of formation of pyrazines is independent of reactant concentration. Using 0.1M solutions of both reactants (sugar and amino acid), concentrations of reactants are quite high, especially relative to pyrazines formed and reactants consumed. Although the rate of formation must be a function of reactant concentration, it may not be apparent due to the relatively high reactant/product ratio, multiplicity of steps in pyrazine formation and competing side reactions. Pigment formation in the Maillard reaction, which is also a multi-step process, has also been shown to exhibit pseudo zero order kinetics by many researchers, including Labuza et al. (28) and Warmbier et al. (29) when reactant concentrations were not limiting for the rate of formation of brown pigment. Loss of reactants has generally been shown to exhibit first order kinetics (29) as well as formation of Amadori compounds, which is essentially a single step process as far as reactants are concerned (30).

Activation energies for alkyipyrazine formation were calculated from the slope of Arrhenius plots, ranging from 27 to 45 kcal/mole (see Table II). Activation energies have been reported for other aspects of the Maillard reaction. The activation energy for browning as measured for pigment production ranges from 15.5 kcal/mole for a glycine-glucose system (31) to 33 kcal/mole for a solid intermediate moisture model food system (29). In the same system, Warmbier et al. (29) reported the activation energies for both lysine and glucose loss to be 25 kcal/mole. The current research indicates that the activation energies for pyrazine formation are higher, suggesting a different rate-controlling step.

The mean activation energies for the dimethylpyrazines are slightly higher than those of the unsubstituted and 2-methylpyrazines. An analysis of variance was performed treating the five sugar-amino acid substrate combinations as a block and pyrazine, 2-methylpyrazine and 2,5-dimethylpyrazine as treatments. No difference in response was found among the substrates at a 95% level of significance. A difference was found to exist among treatments, at an α of 0.05. Duncan's multiple range test was performed to determine which activation energies differed at the same level of significance (0.05). It was found that the

Table I. Regressions for the effect of type of sugar and amino acid on the formation of pyrazines

<u>Model System</u>	<u>Temperature</u>	<u>k (ppm/hr)</u>	<u>k₀ (intercept)</u>	<u>Number of samples</u>	<u>r²</u>
<u>LYSINE-GLUCOSE</u>					
pyrazine	95 C	3.596	0.0596	22	0.994
	85 C	0.490	0.458	22	0.960
	75 C	0.214	0.279	20	0.965
2-methyl-pyrazine	95 C	2.837	-0.104	22	0.995
	85 C	0.422	0.142	22	0.967
	75 C	0.159	0.0910	20	0.941
2,5-di-methyl-pyrazine	95 C	0.186	-0.0457	20	0.995
	85 C	0.0247	-0.00604	22	0.985
	75 C	0.00668	-0.00536	16	0.942
2,3-di-methyl-pyrazine	95 C	0.0229	-0.00569	16	0.948
	85 C	0.00309	-0.00173	18	0.978
	75 C	0.000677	-0.00860	12	0.958
<u>LYSINE-FRUCTOSE</u>					
pyrazine	95 C	1.359	-0.226	20	0.995
	85 C	0.395	-0.0991	22	0.995
	75 C	0.134	-0.103	21	0.963
2-methyl-pyrazine	95 C	1.105	0.301	20	0.995
	85 C	0.388	0.119	22	0.945
	75 C	0.116	-0.0343	21	0.968
2,5-di-methyl-pyrazine	95 C	0.175	0.0488	20	0.945
	85 C	0.0376	0.0211	20	0.951
	75 C	0.00779	0.00570	15	0.935
2,3-di-methyl-pyrazine	95 C	0.0441	0.0127	16	0.954
	85 C	0.117	0.00328	16	0.969
<u>LYSINE-RIBOSE</u>					
pyrazine	95 C	3.488	0.530	21	0.974
	85 C	0.992	0.764	22	0.916
	75 C	0.310	0.747	22	0.882
2-methyl-pyrazine	95 C	5.364	1.380	19	0.957
	85 C	1.768	0.914	22	0.929
	75 C	0.440	0.350	22	0.952

Continued on next page.

Table 1 (cont'd)

<u>Model System</u>	<u>Temperature</u>	<u>k (ppm/hr)</u>	<u>k₀ (intercept)</u>	<u>Number of samples</u>	<u>r²</u>
<u>LYSINE-RIBOSE</u>					
2,5-di-	95 C	0.152	0.0265	18	0.975
methyl-	85 C	0.0440	0.0204	17	0.933
pyrazine	75 C	0.0128	0.00717	18	0.984
2,3-di-	95 C	0.0166	0.00127	17	0.973
methyl-	85 C	0.00427	0.000369	16	0.958
pyrazine					
<u>ASPARAGINE-GLUCOSE</u>					
pyrazine	95 C	0.103	-0.0371	22	0.987
	85 C	0.0422	-0.0385	22	0.941
	75 C	0.00981	-0.0187	22	0.947
2-methyl-	95 C	0.442	-0.215	22	0.992
pyrazine	85 C	0.179	-0.242	22	0.926
	75 C	0.0343	-0.102	22	0.942
2,5-di-	95 C	0.0871	-0.0117	22	0.982
methyl-	85 C	0.0202	-0.00255	22	0.968
pyrazine	75 C	0.00455	-0.0153	14	0.863
2,6-di-	95 C	0.0997	-0.0875	21	0.979
methyl-	85 C	0.0188	-0.0294	11	0.821
pyrazine					
2,3-di-	95 C	0.00231	-0.000192	20	0.992
methyl-					
pyrazine					
<u>ASPARAGINE-FRUCTOSE</u>					
pyrazine	95 C	0.0227	-0.0135	20	0.904
	85 C	0.00653	-0.00631	20	0.881
	75 C	0.00266	-0.00328	20	0.947
2-methyl-	95 C	0.632	-0.331	22	0.927
pyrazine	85 C	0.163	-0.197	22	0.926
	75 C	0.0374	-0.0928	22	0.896
2,5-di-	95 C	0.487	-0.107	22	0.983
methyl-	85 C	0.122	-0.0192	22	0.995
pyrazine	75 C	0.0261	-0.0323	20	0.940
2,6-di-	95 C	0.541	-0.248	20	0.960
methyl-	85 C	0.152	-0.104	20	0.980
pyrazine	75 C	0.0356	-0.0547	18	0.921

activation energies for 2,5-dimethylpyrazine were significantly higher than those of pyrazine and 2-methylpyrazine. However, in performing an analysis of variance the assumption must be made of constant variance for all analyses. This assumption was found to be violated in determining the variances on the regressions from which the activation energies were calculated. One might anticipate these results, since three points were used in the Arrhenius plots. Therefore, further research in this area is necessary to determine the significance of these findings.

Table II. Activation energies for formation of pyrazines in 0.1M sugar-amino acid systems

<u>PYRAZINE</u>	<u>E_a in kcal/mole</u>
lys-glu	35.8
lys-fruc	29.5
lys-rib	30.8
asp-glu	30.0
asp-fruc	27.3
<u>2-METHYLPYRAZINE</u>	
lys-glu	36.6
lys-fruc	28.7
lys-rib	31.9
asp-glu	32.6
asp-fruc	36.0
<u>2,5-DIMETHYLPYRAZINE</u>	
lys-glu	42.3
lys-fruc	39.6
lys-rib	31.5
asp-glu	37.6
asp-fruc	37.3
<u>2,6-DIMETHYLPYRAZINE</u>	
asp-fruc	34.7
<u>2,3-DIMETHYLPYRAZINE</u>	
lys-glu	44.8

The effect of type of amino acid and sugar on the formation of pyrazines was investigated by comparing total yields and relative product distributions of sugar-amino acid systems heated at 95°C for 2 h, as seen in Table III and Figure 3. The type of amino acid reacted had a pronounced effect on total yield of pyrazines produced. A greater yield always resulted with lysine than with asparagine, especially when reacted with glucose. The lysine-glucose system yielded 13.1 ppm total pyrazines while the asparagine-glucose resulted in 0.74 ppm. A system consisting of 0.1M cysteine and glucose, pH 9.0, was also reacted under the same conditions. After 2 h of reacting at 95°C, no pyrazines were detected.

The effect of sugar on total yields of pyrazines was also investigated. In the lysine-sugar systems, total yield was greatest with the pentose, ribose, with 20 ppm total pyrazines produced. Glucose resulted in 13 ppm, versus fructose with 5.7 ppm total pyrazines. With asparagine, the effect of hexoses on

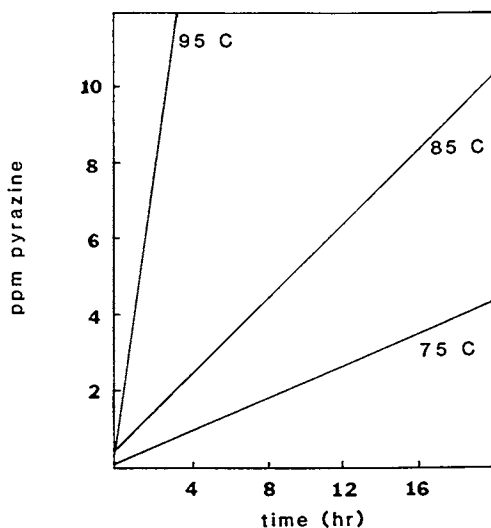


Figure 2. Effect of temperature on the formation of pyrazine

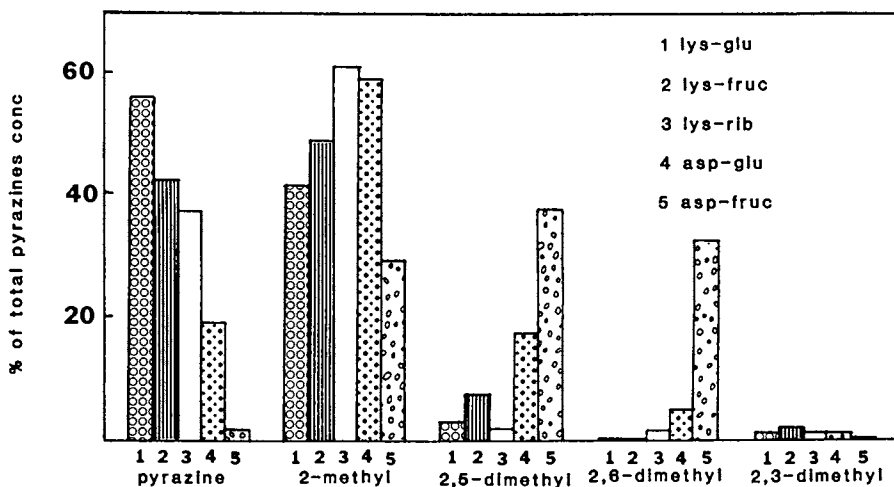


Figure 3. Effect of type of amino acid and sugar on pyrazine distribution - 2 hr at 95°C

total yield showed reverse results, with fructose having a greater total yield than glucose (2.2 ppm versus 0.74 ppm).

Table III. Effect of type of amino acid and type of sugar on pyrazine distributions, 2 h treatment at 95°C

	L-G*	L-F*	L-R* %	A-G*	A-F*
pyrazine	55.8	42.3	36.8	18.7	1.0
2-methylpyrazine	41.5	48.6	61.1	58.8	29.1
2,5-dimethylpyrazine	2.4	7.3	1.6	17.3	37.6
2,6-dimethylpyrazine	—	—	0.3	4.8	32.3
2,3-dimethylpyrazine	0.3	1.8	0.2	0.4	—
TOTAL (ppm)	13.1	5.7	19.9	0.74	2.2

* L = lysine, A = asparagine, G = glucose, F = fructose, R = ribose

The effect of type of amino acid on relative distribution of pyrazines can be seen in comparing lysine-sugar systems with asparagine-sugar systems. Relative yield (percentage of total pyrazines formed) of unsubstituted pyrazine is much greater for lysine than for asparagine with 56% for lysine-glucose versus 19% for asparagine-glucose, and 42% for lysine-fructose versus 1% for asparagine-fructose. For 2-methylpyrazine, no trend was apparent as relative yield was less for lysine-glucose (42%) than for asparagine-glucose (59%) but reversed with a greater relative yield for asparagine-fructose (49%) versus lysine-fructose (29%). Relative yields of the dimethylpyrazines were greater with asparagine versus lysine, with 22% for asparagine-glucose versus 3% for lysine-glucose and 70% for asparagine-fructose versus 9% for lysine-fructose. Under the conditions studied (95°C, 2 h treatment) no 2,6-dimethylpyrazine was detected in measurable quantities in the lysine systems, except with ribose, whereas in the asparagine systems this compound was detected, especially in considerable relative quantities in the asparagine-fructose system. The reverse situation occurred with 2,3-dimethylpyrazine. None was detected in the asparagine-fructose system and only 3 ppb in the asparagine-glucose system versus 30-100 ppb in the lysine systems.

The effect of type of sugar on relative distributions of pyrazines was most pronounced in the asparagine systems. For pyrazine, relative yield was greater with glucose versus fructose, with 56% for lysine-glucose versus 42% for lysine-fructose and 19% for asparagine-glucose versus 1% for asparagine-fructose. For 2-methylpyrazine, relative yields were close in the lysine systems, with 42% for lysine-glucose versus 49% for lysine-fructose. However, in the asparagine systems relative yields were greater with glucose versus fructose, with 59% for asparagine-glucose versus 29% for asparagine-fructose. For dimethylpyrazines, relative yields in both lysine and asparagine systems were always greater with fructose versus glucose,

lysine-fructose with 9% versus lysine-glucose, 3%, and asparagine-fructose with 70% versus asparagine-glucose, 22%. This suggests that relative distributions of pyrazines formed is a function of sugar fragmentation.

The results from these studies compared favorably with the results of van Praag et al. (15) who found the same series of pyrazines resulted, regardless of source of sugar. Also, Koehler et al. (16) found that the source of carbohydrate affects the total yield of pyrazines in ammonium chloride-sugar systems. Yields were greater with fructose versus glucose. Koehler and Odell (22) also found that the ratio of dimethylpyrazine to methylpyrazine differed, depending on sugar reacted.

Shibamoto and Bernhard (18) investigated the effect of sugar source on relative distributions of pyrazines. They found minor differences in relative distributions of pyrazines among the different sugars reacted with ammonium hydroxide. This appears to contradict the results of the current study. They concluded that since glucose is readily transformed to fructose and to a lesser amount of mannose through an enediol reaction intermediate in alkaline solutions, the pathway of pyrazines formation for these three sugars is similar which results in similar pyrazine distributions. The reaction systems which they used consisted of 8M ammonium hydroxide and 1M carbohydrate in water which results in reaction mixtures of extremely high basicity. pH has an effect both on the mutarotation of sugars and enolization/isomerization (32). Although Isbell (32) has demonstrated that D-glucose enolizes at a faster rate than D-fructose and Overend et al. (34) have found that percentages of acyclic conformation of sugars to vary, the extreme conditions in basicity may have acted to negate these differences, so that the net result was similar yields of pyrazines and their distributions. Conditions in the present study were milder at pH 9.0, so differing rates of mutarotation and enolization/isomerization of sugars may have allowed for the formation of different quantities of reaction intermediates, resulting in different total yields of pyrazines as well as distributions. Like the results of the current study, Shibamoto and Bernhard (18) did find pyrazine total yields were greater with pentoses than with hexoses. Unlike their study, the current study employed buffered solutions. This may have had an effect on pyrazine formation.

Burton and McWeeney (35) investigated the stability of sugars in the nonenzymatic browning reaction. They found that the development of chromophores proceeded at a faster rate for pentoses than hexoses and that the mutarotation velocities, as measured by polarographic wave heights, correlated with chromophore production. It seems reasonable to predict greater rates of pyrazine formation with increased rates of mutarotation of sugars. This was seen in the current study with systems containing a pentose (ribose) versus hexoses (glucose and fructose) in reaction with lysine. As far as predicting reactivities of glucose versus fructose, previous studies indicate that this is a bit more complex. Although Burton and McWeeney (35) found ketoses gave greater polarographic waves than the corresponding aldoses (fructose versus glucose), Kato et al. (36) found that fructose initially reacts faster than glucose as

measured by color development, but then the values for glucose surpass fructose. This may be why total pyrazine yields were greater in the lysine-glucose system when reacted for 2 h at 95°C, while the reverse was found in asparagine systems.

With regard to the effect of type of amino acid, Newell et al. (13) found the same series of pyrazines to result regardless of the amino acid reacted. Under the conditions of the current study, 2,6-dimethylpyrazine was detected in the asparagine systems but not in the lysine systems except in reaction with ribose. The other pyrazines detected were similar for all reaction systems. The difference in results here is probably a result of limits of detection. Koehler et al. (16) observed formation of a similar series of pyrazines when reacting glucose with asparagine, glutamine, glutamic acid, aspartic acid and ammonium chloride. However, both total yields and relative product distribution of pyrazines differed as was observed in the current study. They proposed that the distribution of pyrazines produced is a function of the ease of nucleophilic attack of the amino acid on the carbonyls. The current data support this theory.

Koehler and Odell (22) found yields of pyrazines to be greater with asparagine than lysine by a factor of 16. This conflicts with results of the current study, in which yields of pyrazines are always greater with lysine than with asparagine. However, the reaction systems employed in the two studies were different. Koehler and Odell (22) reacted 0.1 mole each of glucose and an amino acid in 100 ml diethyleneglycol and 20 ml of water at 120°C for 24 h. Therefore, pH and water activity of the two systems differed which may have exerted some effect on the reactivities of the amino acids. Also, the unsubstituted pyrazine was not quantified in the previous study. This pyrazine was the major one produced in the current study. Although the α -amino group is most likely the major amino group reactive in the Strecker reaction, the ϵ -amino group will be reactive in sugar-amino condensation, which will eventually result in greater yields of sugar fragmentation products. One would not expect the amide group of asparagine to participate in the Maillard reaction since it is a relatively stable neutral moiety. Therefore, greater yields of pyrazines with lysine versus asparagine seem quite reasonable.

Summary

In this investigation of the effects of types of sugars and amino acids on pyrazine formation, rate of formation best fit pseudo zero order reaction kinetics. Activation energies for pyrazine formation ranged from 27-45 kcal/mole. These values are higher than those reported in the literature for other aspects of the Maillard reaction suggesting a different rate-controlling step. Mean activation energies for dimethylpyrazine formation are slightly higher than those of the unsubstituted and methylpyrazine formation. Both type of amino acid and type of sugar had an effect on total yield and relative distributions of pyrazines.

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Chapter 8

Formation and Aroma Characteristics of Heterocyclic Compounds in Foods

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Heterocyclic compounds have been identified as important volatile components of many foods. The odor strength and complexity of these compounds makes them desirable as flavoring ingredients.

Heterocyclic compounds are primarily formed through nonenzymatic browning reactions. Recent studies of deep-fat fried food flavors led to the identification of pyrazines, pyridines, thiazole, oxazoles and cyclic polysulfides which had long-chain alkyl substitutions on the heterocyclic ring. The involvement of lipid or lipid decomposition products in the formation of these compounds could account for the long-chain alkyl substitutions.

Our knowledge of the chemical composition of food flavors has made considerable progress during the last twenty years. This is mainly due to advances in analytical techniques, such as the coupling of GC with MS and the development of fused-silica capillary columns. Heterocyclic compounds occupy a prominent position among the more than 10,000 compounds occurring in the volatiles of foods. This results from their exceptional sensory properties (1). Heterocyclic compounds contain one or more heteroatoms (O, S and/or N) in rings or fused ring systems.

The majority of heterocyclic compounds are formed through thermal interactions of reducing sugars and amino acids, known as the Maillard reaction. Other thermal reactions such as hydrolytic and pyrolytic degradation of food components (e.g. sugars, amino acids, vitamins) and the oxidation of lipids also contribute to the formation of heterocyclic compounds responsible for the complex flavor of many foodstuffs. Heterocyclic compounds may also be formed enzymatically in vegetables (tomatoes, bell peppers, aspara-

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gus), fruits (pineapple, passion fruit) and during the ripening of cheese.

Recent studies in our laboratory showed that lipids may be directly associated with the Maillard reaction in the formation of some heterocyclic compounds. The effect of lipids on the formation of heterocyclic compounds in a model Maillard reaction has also been reported by Mottram and Whitfield (2).

This paper discusses the formation and aroma characteristics of selected classes of heterocyclic compounds important to the flavor of foods, especially deep-fat fried foods.

Pyrazines

Alkylpyrazines have been recognized as important trace flavor components of a large number of cooked, roasted, toasted and deep-fat fried foods (3). As a rule, alkylpyrazines have a roasted nut-like odor and flavor. Formation pathways for alkylpyrazines have been proposed by numerous researchers (4, 5, 6). Model studies suggest that they are minor products of the Maillard reaction.

The recent identification of 2-heptylpyrazine in french fried potato flavor, and 2-methyl-3(or 6)-pentylpyrazine, 2-methyl-3(or 6)-hexylpyrazine and 2,5-dimethyl-3-pentylpyrazine in a heated and extruded corn-based model system, deserve special attention (7, 8, 9). These alkylpyrazines have a long-chain substitution on the pyrazine ring. Only the involvement of lipids or lipid-decomposition products in the formation of these compounds could account for the long-chain alkyl substitution on the pyrazine ring. A mechanism for the formation of 2,5-dimethyl-3-pentylpyrazine was proposed and is shown in Figure 1. 3,6-Dihydropyrazine, formed by the condensation of aminoketones, reacts with pentanal, a lipid oxidation product, and results in the formation of 2,5-dimethyl-3-pentylpyrazine. The possible reactivity of 3,6-dihydropyrazine with carbonyl compounds has been discussed by Flament (10). Rizzi (11) reported the formation of 2,5-dimethylpyrazine and 2,6-dimethylpyrazine in the reaction of 1-hydroxy-2-propanone (acetol) with ammonium acetate under mild conditions and acidic pH. The proposed mechanism was also supported by our identification of 2,5-dimethyl-3-pentylpyrazine and 2,6-dimethyl-3-pentylpyrazine as the major products when pentanal was added to a mixture of acetol and ammonium acetate and reacted at an elevated temperature (100°C). 2-Heptylpyrazine has green, waxy and earthy notes and could be an important contributor to the flavor of french fried potatoes or other fried food systems.

Various isopentyl-substituted pyrazines, such as 2-isopentyl-3-methylpyrazine, 2-isopentyl-5-methylpyrazine, 2-isopentyl-6-methylpyrazine, 2-isopentyl-5,6-dimethylpyrazine, 2-isopentyl-3,5-dimethylpyrazine and 2-isopentyl-3,6-dimethylpyrazine were identified from the thermal reaction of glucose and leucine (12). The formation mechanisms for these compounds may also involve the reaction of 3,6-dihydropyrazine with isovaleraldehyde, the Strecker aldehyde of leucine. Kitamura and Shibamoto (13) described 2-isopentyl-5,6-dimethylpyrazine as having a caramel-like, coffee and sweet aroma. Although isopentyl-substituted pyrazines have not yet been reported in cocoa, they could, if present, be very important contributors to that characteristic aroma.

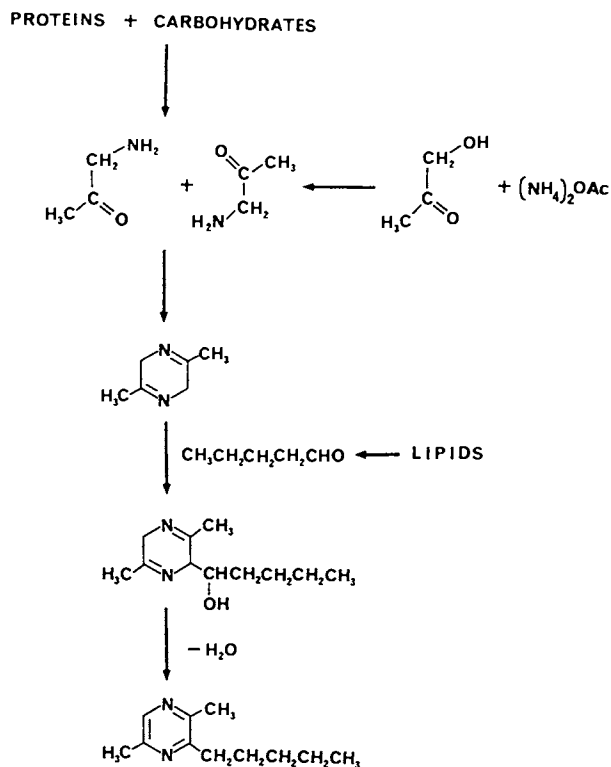


Figure 1. Mechanism for the formation of 2,5-dimethyl-3-pentylpyrazine.

Although most of the alkylpyrazines are formed through thermal interactions of components in food, methoxy-substituted pyrazines are mainly derived from biosynthetic pathways. 2-Isobutyl-3-methoxy-pyrazine isolated from bell pepper by Buttery et al. (14) is one of the most significant flavor compounds discovered. This characteristic bell pepper aroma compound has an extremely low odor threshold of 0.002 ppb in water (15).

Pyridines

The occurrence of pyridines in food has been reviewed (16). 2-Alkyl-pyridines were proposed to form from the corresponding unsaturated n-aldehydes with ammonia upon heat treatment (17, 18). Table I lists pyridines identified in the volatiles of fried chicken (19) and french fried potatoes (7).

Table I. Pyridines Identified in Fried Chicken and French Fried Potato Flavor

Compound	Fried Chicken	French-Fried Potato
Pyridine	+	
2-Methylpyridine	+	
2-Ethylpyridine		+
3-Ethylpyridine	+	
4-Ethylpyridine	+	
2-Acetylpyridine		+
2-Methyl-5-ethylpyridine	+	
2-Ethyl-3-methylpyridine	+	
2-Butylpyridine	+	+
2-Pentylpyridine	+	+
2-Heptylpyridine		+
2-Pentyl-3,5-dibutylpyridine		+
2-Isobutyl-3,5-dipropylpyridine	+	

2-Pentylpyridine was identified in both fried chicken and french fried potato flavors. This compound has a strong fatty and tallow-like odor and was the major product in the volatiles generated from the thermal interaction of valine and linoleate (20). It is postulated to form through the reaction of 2,4-decadienal and ammonia (20). The reaction of 2,4-decadienal with either cysteine or glutathione (γ -glu-cys-gly) in aqueous solution at high temperature (180°C) yielded 2-pentylpyridine as the major product (Zhang, Y. and Ho, C.-T., Rutgers University, unpublished data). The amount of 2-pentylpyridine generated in the 2,4-decadienal/glutathione system did not differ significantly from that in the 2,4-decadienal/cysteine system. It is possible that the amino group from amino acids, or peptide, condenses directly with the aldehydic group of 2,4-decadienal and is then followed by an electrocyclic reaction and aromatization to form 2-pentylpyridine (Figure 2).

2-Isobutyl-3,5-diisopropylpyridine was identified in fried chicken and has a roasted cocoa-like aroma (21). Figure 3 shows the mechanism for the formation of this compound as proposed by Shu et

al. (22). It involves the reaction of aldehyde and ammonia at high temperatures and is known as the Chichibabin condensation. 2-Iso-butyl-3,5-diisopropylpyridine has also been identified in a glucose/leucine model system possessing a cocoa-like aroma (23). 2-Pentyl-3,5-dibutylpyridine was identified in french fried potato flavor and presumably formed through a Chichibabin reaction involving ammonia and hexanal, an abundant thermal oxidative decomposition product of lipids.

Thiazoles

Thiazoles are a class of compounds possessing a five-membered ring with sulfur and nitrogen in the 1 and 3 positions, respectively. The potential for thiazole derivatives as flavorants is evident from the work of Stoll et al. (24) who found the strong nut-like odor of a cocoa extract to be due to a trace amount of 4-methyl-5-vinylthiazole. Since then, numerous thiazoles have been identified in food flavors.

The exact origin of thiazoles remains a mystery. They might form through the thermal degradation of cystine or cysteine (25, 26), or by the interaction of sulfur-containing amino acids and carbonyl compounds (27, 28). Thiazoles have been identified as volatile components of thermally degraded thiamine (29).

Aroma properties of some alkylthiazoles have been reviewed (30). The most typical alkylthiazole is probably 2-isobutylthiazole. This compound was isolated from tomato flavor and was described as having a strong green odor resembling that of tomato leaf (31). When added to canned tomato puree or paste at levels of 20 to 50 ppb, 2-isobutylthiazole develops an intense fresh tomato-like flavor. Most of the alkylthiazoles are described as green, nutty and vegetable-like (32, 33).

Table II lists alkylthiazoles identified in fried chicken flavor (19) and french fried potato flavor (7).

Several of the alkylthiazoles identified in french fried potato flavor, such as 2,4-dimethyl-5-propylthiazole, 2,4-dimethyl-5-pentylthiazole, 2-butyl-4-methyl-5-ethylthiazole and 2-butyl-4-propylthiazole have a strong characteristic sweet, sulfury and green aroma (33). This aroma characteristic is quite distinctive and is present in a large number of the fractions generated from the gas chromatographic fractionation of the french fried potato flavor isolate. It is probably an important part of the total french fried potato flavor.

2-Pentyl-4-methyl-5-ethylthiazole has a strong paprika pepper flavor and 2-heptyl-4,5-dimethylthiazole has a strong spicy flavor. 2-Octyl-4,5-dimethylthiazole has a sweet fatty aroma (33). They are probably important contributors to the flavor of fried foods. These thiazoles and other thiazoles identified have long-chain alkyl substitutions on the thiazole ring. The involvement of frying fat or fat decomposition products in the formation of these compounds is again suggested.

Thiazolines, a reduced form of thiazoles, have also been reported to occur in foods, mainly cooked beef. 2,4,5-Trimethyl-2-thiazoline was identified in beef broth (34). 2,4-Dimethyl-3-thiazoline found in cooked beef aroma was reported to have a nutty, roasted and vegetable aroma (35).

Table II. Alkylthiazoles Identified in Fried Chicken and French Fried Potato Flavor

Compound	Fried Chicken	French-Fried Potato
Thiazole	+	
2-Methylthiazole	+	
2,4,5-Trimethylthiazole	+	+
2-Methyl-4-ethylthiazole	+	
2,4-Dimethyl-4-ethylthiazole	+	
2-Ethyl-4,5-dimethylthiazole		+
2-Isopropyl-4-methylthiazole		+
2-Propyl-4,5-dimethylthiazole	+	+
2-Isopropyl-4,5-dimethylthiazole		+
2,5-Dimethyl-4-butylthiazole	+	+
2-Isopropyl-4-ethyl-5-methylthiazole	+	+
2-Butyl-4,5-dimethylthiazole	+	+
2-Isobutyl-4,5-dimethylthiazole		+
2,4-Dimethyl-5-pentylthiazole		+
2,4-Diethyl-5-propylthiazole		+
2-Butyl-4-methyl-5-ethylthiazole	+	+
2-Butyl-4-propylthiazole		+
2-Pentyl-4,5-dimethylthiazole	+	+
2-Butyl-4-propyl-5-methylthiazole		+
2-Pentyl-4-methyl-5-ethylthiazole		+
2-Pentyl-5-propylthiazole		+
2-Hexyl-4,5-dimethylthiazole	+	+
2-Heptyl-4,5-dimethylthiazole	+	+
2-Heptyl-4-ethyl-5-methylthiazole	+	+
2-Octyl-4,5-dimethylthiazole	+	+
2-Octyl-4-methyl-5-ethylthiazole		+

Oxazoles

Oxazoles are characterized by possessing a five-membered ring with oxygen and nitrogen in the 1 and 3 positions. The occurrence of oxazoles in food flavor has been reviewed (36). Recently twenty-four alkyloxazoles were identified in the volatile compounds from french-fried potatoes (37). This represents the largest number of oxazoles reported in a food system. Like the alkylthiazoles, several of the alkyloxazoles identified, such as 2-pentyl-4,5-dimethyl-oxazole, 2-pentyl-4-methyl-5-ethyloxazole, 2-hexyl-4,5-dimethyloxazole and 2-hexyl-4-methyl-5-ethyloxazole, have long-chain alkyl substitutions at the 2-position of the oxazole ring. Lipids or lipid decomposition products could be involved in the formation of these long-chain alkyl substituted oxazoles.

Oxazoles have a wide range of aroma characteristics. As an example, Table III lists the aroma characteristics of some alkyloxazoles identified in fried bacon flavor (Lee, K. N., Rutgers University, unpublished data).

Table III. Odor Description of Alkyloxazoles Identified in Fried Bacon Flavor

Oxazole	Odor Description
5-butyloxazole	very strong, bacon fatty, Animalic, aged meat-like
2,5-dimethyl-5-propyloxazole	green, flowery, sweet
2-methyl-5-pentyloxazole	sweet, strong floral, fatty-waxy
2,5-dimethyl-4-butyloxazole	fresh acidic green, pickle-like
2,5-dimethyl-5-butyloxazole	dry herbal, seasonal herbal
2-isopropyl-4-ethyl-5-methyloxazole	sweet, fruity
2-phenyl-5-ethyloxazole	indole-like, phenolic

It is interesting to note that 5-butyloxazole has a very distinct bacon-fatty aroma and could be an important flavor constituent of fried bacon. 5-Pentyloxazole also possesses a similar aroma characteristic. Both 5-butyloxazole and 5-pentyloxazole have no alkyl group on carbon 2 or 4 of the oxazole ring. When a methyl group is substituted on carbon-2 (e.g., 2-methyl-5-pentyloxazole), the fatty aroma decreases and a sweet-floral aroma becomes more characteristic. The sweet-floral character is further enhanced by additional methyl substitution on carbon-4 (38).

2-Pentyl-4-methyl-5-ethyloxazole was identified in french-fried potato flavor and has a strong buttery, sweet and lactone-like flavor. It is probably an important contributor to the fried food aspect of french-fried potato flavor (37).

Oxazoles and thiazoles possessing comparable alkyl groups were reported to have significant aroma similarities (33). Buttery et al. reported that some 4,5-dialkylthiazoles possessed potent bell pepper aroma (39). The most potent one, 4-butyl-5-propylthiazole, was reported to have a flavor threshold of 0.003 ppb in water. Ho and Tuorto (40) synthesized several 4,5-dialkyloxazoles and found them to have a green, vegetable-like aroma. 4-Butyl-3-propyloxazole has a strong bell pepper aroma and a flavor threshold of 0.1 ppm in water. 5-Pentylthiazole also had strong fatty and sweet aromas reminiscent of 5-pentyloxazole. 2-Pentyl-5-methylthiazole was judged to have a fermented vegetable-like aroma. The corresponding 2-pentyl-5-methyloxazole was described as acidic and sweet with a flowery afternote (38).

Figure 4 shows a proposed mechanism for the formation of 2,4,5-trimethyloxazole and 4,5-dimethyloxazole from the Strecker degradation of cysteine with 2,3-butanedione (41).

3-Oxazolines, the reduced form of oxazoles, also have important sensory properties. The first report of a 3-oxazoline was made by

Chang et al. (42). They isolated and identified 2,4,5-trimethyl-3-oxazoline in boiled beef. This compound was described as having a "characteristic boiled beef aroma". Mussinan et al. (35) identified oxazolines and no oxazoles in their beef system. Peterson et al. (43) reported on the volatiles of canned beef stew. Both 2,4,5-trimethyloxazole and 2,4,5-trimethyl-3-oxazoline were present. The relative concentration of 2,4,5-trimethyloxazole was medium while for 2,4,5-trimethyl-3-oxazoline was extra high. Lee et al. (44) identified 2-methyl-3-oxazoline, 2,4-dimethyl-3-oxazoline and 2,4,5-trimethyl-3-oxazoline in the volatiles of roasted peanuts. The latter two 3-oxazolines were also identified in the volatiles of fried chicken (19).

Trithiolanes

Trithiolanes have received increasing attention since the identification of diastereomeric 3,5-dimethyl-1,2,4-trithiolane in the volatiles of boiled beef (42). The parent 1,2,4-trithiolane is a component of Shiitake mushrooms (45) and red algae (27). In addition to 3,5-dimethyl-1,2,4-trithiolane, Kubota et al. (46) identified 3-methyl-5-ethyl-1,2,4-trithiolane and 3,5-diethyl-1,2,4-trithiolane in both *cis* and *trans* forms in boiled Antarctic Gulls. Both compounds were described as garlicky. Flament et al. (47) reported the identification of 3-methyl-5-ethyl-1,2,4-trithiolane and 3-methyl-5-isopropyl-1,2,4-trithiolane in a commercial beef extract.

3,5-Diisobutyl-1,2,4-trithiolane was identified in the volatiles isolated from fried chicken (21). This compound has been reported to possess roasted, roasted-nut, crisp bacon-like and pork rind-like aromas and flavors (48). In addition to 3,5-dimethyl-1,2,4-trithiolane and 3,5-diisobutyl-1,2,4-trithiolane, two long-chain alkyl substituted trithiolanes, namely, 3-methyl-5-butyl-1,2,4-trithiolane and 3-methyl-5-pentyl-1,2,4-trithiolane, were reported to be present in fried chicken flavor (49). Along with 3,5-dimethyl-1,2,4-trithiolane, 3-methyl-5-ethyl-1,2,4-trithiolane, 3-methyl-5-propyl-1,2,4-trithiolane and 3-methyl-5-butyl-1,2,4-trithiolane are reported to be important flavor components of Chinese stewed pork (50).

A mechanism has been reported for the formation of trithiolane from the reaction of aldehydes with hydrogen sulfide (51). The identification of 3-methyl-5-butyl-1,2,4-trithiolane and 3-methyl-5-pentyl-1,2,4-trithiolane in food flavor suggests that pentanal and hexanal were involved in the formation of these compounds (Figure 5). Pentanal and hexanal are major thermal and oxidative decomposition products of lipids.

Summary

Heterocyclic compounds, especially those which contain nitrogen and sulfur atoms, possess potent sensory qualities at low concentrations. They are formed in foods by thermal decomposition and interaction of food components. The identification of many long-chain alkyl substituted heterocyclic compounds suggests that their formation mechanisms directly involve lipids or lipid decomposition products.

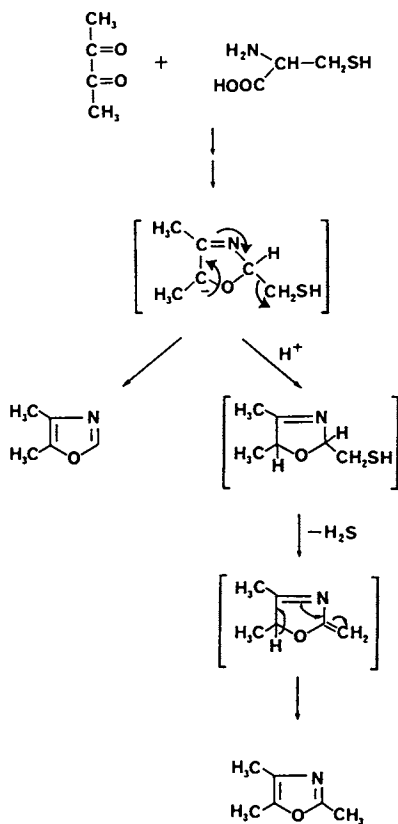


Figure 4. Mechanism for the formation of 2,4,5-trimethyloxazole.

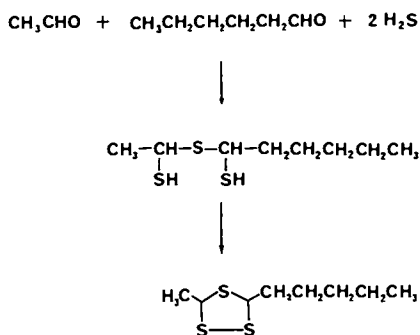


Figure 5. Mechanism for the formation of 3-methyl-5-pentyl-1,2,4-trithiolane.

Acknowledgments

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Chapter 9

Natural Flavors Produced by Biotechnological Processing

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The world market for flavors is growing steadily with a distinct trend towards 'natural' compounds. Biotechnology offers many advantages over traditional extraction of botanical materials for flavors production. These include highly specific end product generation (e.g. optically-active compounds), high yields and purity, along with guaranteed supply. Commercial exploitation of biotechnology in this area not only relies upon technical advances but as well on satisfying certain regulatory considerations. These aspects are highlighted using examples of whole cell microbial culture and isolated enzyme systems.

A recent Japanese report predicts that products from the food and beverage industry will top the list of biotechnology products in sales by the year 2000 (1). While demand for food products can fluctuate, particularly at the bulk end of the market, certain high priced products such as flavor/aroma compounds are experiencing a constant increase in demand (2). Flavoring compounds, substances gratifying taste and smell, represent 10-15% by weight of world-use food additives, which amounts to 25% of the value of the total food additives market (3). Recently, it has been estimated that 5,000-10,000 natural flavor compounds exist (4) with 4,300 having been identified (5).

The market for flavors is anticipated to grow at an annual rate of about 30% (6). An aging population and the associated diminution of taste acuity among older persons has resulted in a need for products with more intense flavors (7). Other market demands for a greater

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variety of flavored products has also increased flavor material consumption. A recent 'sensory audit' conducted by a leading food processor revealed that consumers were most concerned about 'freshness' and 'naturalness' in products they buy (7). This trend can be ascribed to increasing health and nutrition conscious life-styles which has encouraged the development of natural food products (8). One way food processors have responded to this trend towards 'naturalness' is an increased use of natural food flavorants found in plants (9). Unfortunately, these raw materials are subject to various problems (8). Often, the desired flavor component is in low concentration and therefore extraction from botanical material is expensive. As well, the supply of the materials is subject to seasonal variation and to the vagaries of the weather, which significantly affects the yield and quality of the flavor. The supply is also influenced by the socio-political stability of the producing regions. The industry, if it is to meet consumer demand effectively, needs competitive new sources of natural flavors, and biotechnology has become a necessary solution.

NATURAL FLAVORS. The meaning of the term 'natural' may vary between different social communities. In the United States the Code of Federal Regulations (CFR 101.22.a.3) defines the term 'natural flavor' to include not only animal or plant derivatives but also includes enzymic and fermentative processes. A natural flavor, according to the U.S. FDA guidelines set down in 1958, must be produced from natural starting materials and that the end-product must be identical to something already known to exist in nature. Thus, biocatalytic, but not chemical, transformation of natural substances can be legally labelled as natural.

Application of Biotechnological Processing for Natural Flavors-Production

Biotechnological approaches for production of flavors have the potential to alleviate the problems outlined with traditional botanical-derived processing routes. Additionally, other benefits can be expected which should serve to control costs, supply and consistency.

Food technologists have had much experience with the use of industrial microorganisms and enzymes in traditional processes (bread and cheese making, alcohol fermentation). Numerous early reports, some dating back to the early 1900s, realized the potential of microorganisms for flavour/aroma production. However a limited knowledge base prevented the application of many biotechnological routes for flavor production. More recently, the level of knowledge pertaining to metabolic pathways and their regulatory mechanisms has allowed for

more control through physiological and/or genetic manipulations. With rapid advances occurring in genetic engineering and fermentation technology, the area of biotechnological flavors production is sure to benefit.

There has been much work conducted recently in the area of plant cell culture, or phytoproduction, especially where the product is a plant-unique mixture of individual flavor substances such as vanilla extract of which vanillin is the major component. As well, the possibility of genetically engineering improved varieties of plants for high yield and consistent quality products is of considerable interest especially for more complex plant-unique flavors. Many flavor compounds are secondary metabolites for which a detailed understanding of their production is not well understood. Presently more knowledge exists in microbial metabolism relative to plant biosynthetic pathways and therefore has resulted in more successful development of microbial-based flavor bioprocessing. As well, scale-up of microbial cultures and isolated enzymes has become relatively common practice while the translation of plant cell culture to large commercial scales is not yet well established. This review will focus on the microbial whole cell and isolated enzyme systems for flavor production.

MICROBIAL WHOLE CELL AND ENZYMIC SYSTEMS: A number of single flavour substances have been identified which are associated with particular flavors (10): 2-isobutylthiazole with tomato flavor; methyl- and ethyl-cinnamates with strawberry flavor; methyl anthranilate with grape flavor; and benzaldehyde with cherry flavor. Single flavor components such as these should be amenable to microbial production from nutrients via multienzyme steps or enzymic production from appropriate precursors.

An important attribute of microbial whole cell biocatalysts is the ability to synthesize products de novo from relatively inexpensive nutrients such as simple sugars. Citric acid, produced by the fungus Aspergillus niger, is perhaps the best known flavor compound. Another fungus Trichoderma reesei can produce a strong coconut-like impression which is the result of the formation of 6-pentyl- α -pyrone (chemical synthesis requires seven steps). The yeast Sporobolomyces odorus can produce the lactones 4-decanolide and cis-6-dodecenen-4-olide, responsible for a peach-like impression (11). Apart from 'fruity' flavors there seems to be a growing interest in the production of natural flavor chemicals related to "toasted" or cooked meat flavors for application to microwave products (12). It has been shown that a mixture of certain sugars and dihydroxyacetone (derived from a fermentation) give an

improvement in browning and taste due to the development of roasting and aromatic compounds (13).

Recently we have found that the yeast Candida utilis can convert glucose, and other fermentable sugars, to ethyl acetate (14). Ethanol can also be converted directly to ethyl acetate or acetaldehyde by this yeast (15) depending upon the concentration of ethanol provided in the medium (Figure 1). Ethyl acetate is important for its use in 'fruity' flavors which acetaldehyde has an important application in providing 'freshness' to many products including fruit-based drinks (16). Both conversions operate when iron limiting conditions are imposed and they demonstrate that relatively simple physiological manipulations can result in marked changes in metabolism. This yeast is capable of 'aerobic' fermentation and readily forms ethanol from fermentable sugars. Under iron-sufficient aerobic conditions C. utilis produces biomass and is currently used commercially for this purpose (single cell protein). The imposition of iron-limited conditions results in the tricarboxylic acid (TCA) cycle being severely rate limited (Figure 2). Certain iron-requiring enzymes in the TCA cycle including succinate dehydrogenase and aconitase become activity limited. Under iron-limitation the intracellular level of acetyl-CoA accumulates which in the presence of ethanol allows for increased ethyl acetate formation via an alcohol transferase mechanism. Through the same metabolic scheme, when the level of ethanol is elevated above approximately 3.5% (w/v), acetaldehyde accumulates. As the rate of oxidation of ethanol to acetaldehyde exceeds that of the oxidation of acetaldehyde to acetic acid, the aldehyde begins to accumulate. This intracellular accumulation forward inhibits acetyl-CoA synthetase which results in a reduction in ethyl acetate formation and a change in product distribution to acetaldehyde. Apart from producing two important flavor substances, the spent yeast itself can be used post-processing in natural flavour applications. Candida utilis can be used for imparting 'savory' or 'meaty' flavors as it is classified by the U.S. FDA as GRAS (Generally-Recognized-As-Safe) and is one of only three yeasts allowable for this purpose in human food products (17).

Another important attribute of whole cell/enzyme systems is for the production of optically-active compounds. As early as the 1960s, the synthesis of optically active gamma- and delta-lactones by microbiological reduction was demonstrated (18). Production of optically-pure L-glutamate for use in the flavor enhancer MSG by microbial means is another testimonial to this potential of biotechnology.

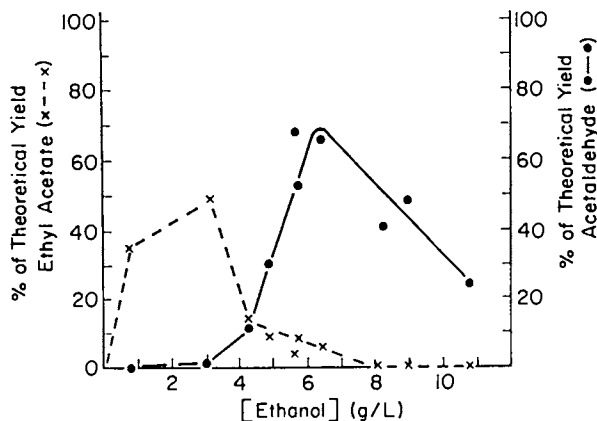


Fig. 1. Change in product distribution from ethyl acetate to acetaldehyde of *Candida utilis* due to increasing levels of added ethanol.

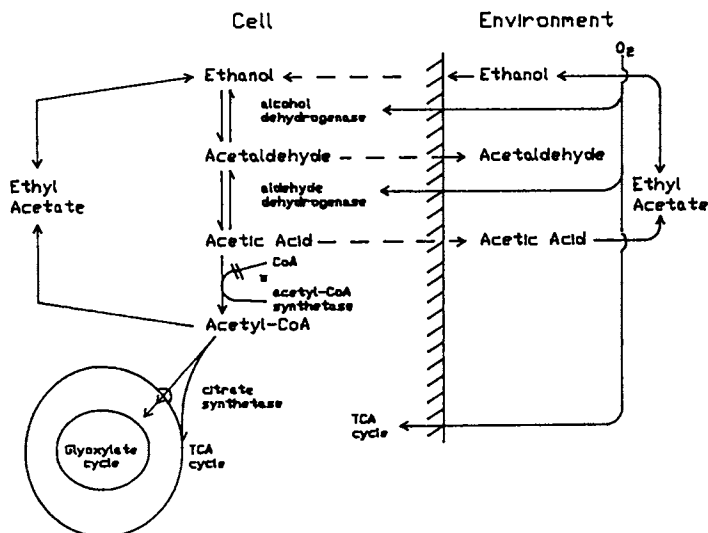


Fig. 2. Pathway of ethanol utilization and ethyl acetate or acetaldehyde production by *Candida utilis*. TCA cycle activity inhibited under iron-limited conditions. Acetyl-CoA synthetase forward inhibited by acetaldehyde accumulated when elevated levels of ethanol ($\geq 3.5\%$ w/v) present in the medium.

Transformation of precursor compounds to flavors can also readily take advantage of biotechnological processing. For example, the sesquiterpene valencene, readily available from orange oil but of low commercial value, can be transformed by certain bacteria (18) to the higher value sesquiterpene, nootkatone (grapefruit citrus flavor) currently valued at about \$4200/kg (19) up from \$1300-1600 a year earlier (synthetic \$700/kg). Another biotransformation having significant impact on the citrus juice industry is the ability of *A. niger* to convert the bitter component naringin to the non-bitter form naringenin (20). Recent consumer demand for grapefruit-based flavors is driving the price up for naringin (currently priced at \$55/kg and rising) which could be converted biologically to an even higher priced non-bitter form. An area where biotransformations would improve product yields, and simultaneously result in an optically-active end product, involves L-menthol production. Currently the market for this terpene is approximately 3000 tons/y and relies upon traditional extraction of plant materials. In the peppermint plant, oil 'maturation' occurs between the initial flowering and full bloom, during which time only a small portion (40%) of L-menthone is converted to L-menthol. It has been proposed that L-menthone could be extracted at peak levels from the plant and subsequently converted via dehydrogenase activity of various microorganisms. Various systems employing the bacterium *Pseudomonas putida* and the yeast *Rhodotorula minuta* have resulted in L-menthol with 100% optical activity (20). The production of flavor complexes where possibly hundreds of individual flavor substances contribute to an overall flavor impression is highly suited to biotransformation. To create the same complex flavor by the addition of individual flavor components would be very difficult if not impossible. As a result, the exogenous addition of whole cells and/or enzymes to accelerate the flavor maturation process of a number of food products is rapidly attaining commercial reality. Cheese ripening or aging is an area of intense research and should lead to significant cost reductions. In the U.S. alone over 1 billion kilograms of Cheddar-type cheese is produced annually. Normally, the aging process takes place over a period of between 3-9 months at a cost of 1-2 cents/kg.month. The aging process involves primarily the action of endogenous biocatalysts which transforms a relatively bland elastic mass to a well-bodied cheddar cheese. Research has shown that two key enzymic mechanisms involving lipase and protease are critical to cheese aging (21). The lipase preferentially hydrolyses triglycerides, yielding C_6-C_{10} free fatty acids while the protease provides a balanced flavor development through proteolytic cleavage. A number of products are now available commercially for accelerated cheese

ripening including a lipase/protease preparation derived from Aspergillus oryzae. Lipases from Candida cylindraceae (rugosa) and Mucor miehei show different specificity for fatty acid chain lengths and have been used to generate different cheese flavor complexes.

Apart from producing flavor substances or complexes, biocatalysts could facilitate current extraction processes. A Japanese process uses fungal cellulase enzymes to enhance juice extraction from oranges without disrupting the rind oil glands which contain 'off' flavors and bitterness. Cellulases and pectinases have also been used to degrade plant cell walls to release more oil from oil seeds during processing. The use of 500-1000 g of enzyme per ton of seeds at temperatures of 30-50°C can produce a 2-6% increase in oil yield (22).

NOVEL FLAVOR BIOPROCESSING SYSTEMS. Typically the systems described above are operated in aqueous environments. It has recently become evident that many enzymes (23) and even certain whole cells (24) can function in apolar or organic solvents such as hexane. Although the above systems are referred to as 'non-aqueous', technically, the enzymes have to be in a biphasic system (organic solvent/water) with the aqueous component being present in low amounts, even down to a monomolecular layer on an enzyme. This knowledge will certainly benefit biotechnological production of flavor production for a number of reasons. Since many flavor compounds or their precursors have limited water solubility, the use of apolar solvents rather than water could allow for more efficient conversions due to better enzyme/substrate interaction. In addition, some reactions may only occur to a significant degree in a more apolar environment such as esterification with lipase enzymes (25). Normally, in the case of a lipase-based conversion, esters are hydrolytically converted to the acid and alcohol moieties. In certain apolar solvents this reaction can be reversed owing to the preferred partitioning of the ester away from the enzyme thereby 'pulling' the reaction (Figure 3). More detailed discussions on the use of organic media for biocatalysis can be found elsewhere (26).

We have conducted studies related to the production of flavor esters using immobilized lipase from the yeast C. cylindraceae in a non-aqueous system (25, 27). The lipase from this microorganism was found to have a broad range specificity and was useful to produce a number of commercially important esters including ethyl butyrate, isobutyl acetate and isoamyl acetate (Table I). The studies focussed on the production of ethyl butyrate, useful in pineapple-banana flavors, owing to its large market demand of over 140,000 kg annually (28) and selling price for the natural ester ranging upwards of

\$150/kg. The system was found to produce significant amounts of this ester and as well had an excellent operational stability (upwards of a month operation).

Table I. Ester production by immobilized C. cylindracea lipase using a range of substrates. The molar concentration is that of ester in the heptane phase after 24 h. Percent molar conversion is that of initial acid to ester after 24 h

ESTER	(M)	CONVERSION
Ethyl propionate	0.19	76
Ethyl butyrate	0.25	100
Ethyl hexanoate	0.09	44
Ethyl heptanoate	0.21	84
Ethyl octanoate	0.26	100
Ethyl laurate	0.13	52
Ethyl isobutyrate	0.18	72
Ethyl isovalerate	0.01	3
Isobutyl acetate	0.06	25
Isoamyl acetate	0.06	24
Isoamyl butyrate	0.22	91

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The key finding was the requirement for intermittent hydration of the immobilized enzyme for long term operational stability (Figure 4). Without this hydration protocol, upwards of 90% of the initial activity was lost after only 2 cycles (ca. 48 h).

Another even more recent advance for flavor processing involves the use of supercritical fluids. Supercritical fluids, including supercritical CO₂, created under elevated pressures, exist in a nebulous region between that of a liquid and that of a gas. The ability to continuously adjust the solubility of the desired component to be extracted by modification of pressure and/or temperature has led to this technology being quickly adopted by food and flavor/fragrance industries (decaffeination, oil extraction, etc.). As well, the concern over the toxicity of certain solvents, has encouraged the search for an alternative extractive process. Supercritical CO₂ has been the 'solvent' of choice by most flavor industries employing this technology since it is inert, nonflammable, nonexplosive and leaves no residue in the final product. Moreover CO₂ is readily available in large quantities and high purity and of great importance it is probably the next cheapest solvent to water (under 10 cents/kg). Additionally, as the critical temperature of CO₂ is only

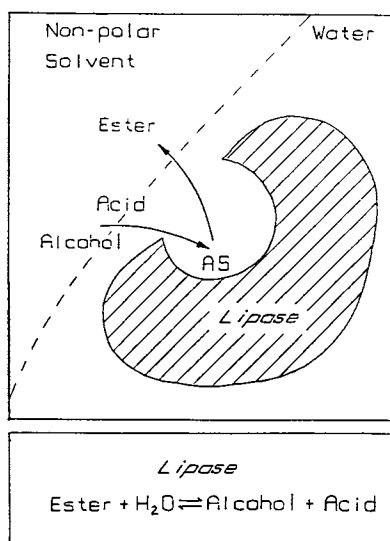


Fig. 3. Production of esters in a non-aqueous lipase system. Water present in low levels around enzyme. Reversal of equilibrium occurs to favor formation of esters. Active site = AS.

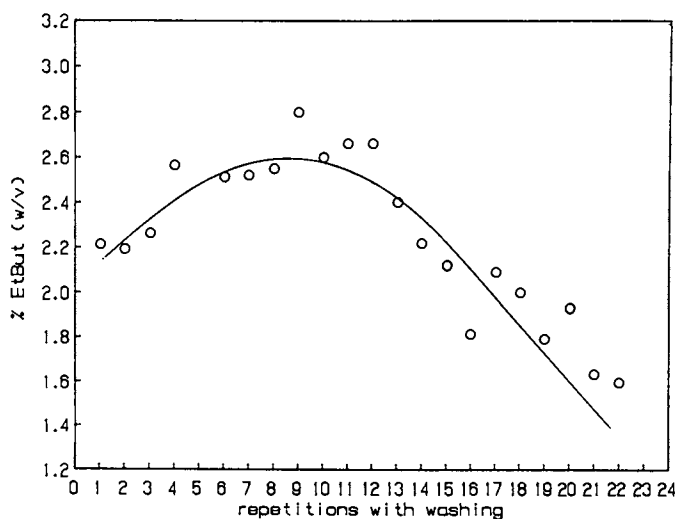


Fig. 4. Repeated use of immobilized lipase in non-aqueous system. Between each repetition the immobilized enzyme adsorbed to a silica gel support matrix was washed with water. Fresh substrate (ethanol, butyric acid) was added in hexane. One cycle represents ca. 24 h.

31°C at 74 bar pressure, thermally labile flavor components such as jasmine can be readily extracted from the botanical source. For the most part, it is these attributes described above that the flavor industry has found attractive for flavor compound extraction that has led biotechnologists to adapt this technology to biocatalysis. Pioneering studies involving the use of lipase in supercritical CO₂ have demonstrated, as detailed above for solvent-based non-aqueous systems, that hydrolytic reactions can be suppressed and synthesis can be significantly enhanced (28, 29). One group (29) found that the reaction rate in this type of system was upwards of 270 times that (measured in more product/mole enzyme.second) compared with an aqueous system. Undoubtedly, more novel biotechnological process routes will become available and as illustrated by these examples, even once considered 'extreme' environments could facilitate natural flavor production.

REGULATORY CONSIDERATIONS-AN UPDATE

In the U.S., FEMA (Flavor and Extract Manufacturers' Association) is in the process of drafting new guidelines for natural flavor/aroma chemicals and accepted processing methods (12). Apart from the accepted use of fermentation, heat reactions and enzymes, it is expected that inorganic acids and bases will be allowable for use as catalysts. Obviously this could allow for production of certain natural flavors, in the absence of enzymes, under appropriate conditions, however the requirement for natural substrates will remain.

Although regulatory agencies, such as the FDA, are trying to keep pace with this rapidly advancing technology many potential bottlenecks and 'grey' areas persist primarily in interpretation of the regulatory requirements. The FDA states that its policies are sufficiently comprehensive to apply to those involving biotechnology. Although, statements (30) such as "...the use of new microorganisms found in a food such as yogurt could be considered a food additive. Such situations will be evaluated case-by-case..." could have significant ramifications. For example, if a new microorganism, possibly manipulated using rDNA technology, is added to a food for flavor improvement, a food additive petition (FAP) could be necessary. This would add much to product development costs and possibly delay effective early market penetration. Normally a GRAS (Generally-Recognized-As-Safe) status for a food substrate allows for immediate use in food products without regulatory clearance. A major concern is that the use of rDNA techniques could cause a substrate, that was normally classified as GRAS, to be seen as a food

additive thereby necessitating regulatory clearance before use (31). The FDA has indicated that the use of an animal food substance prepared by rDNA technology must be approved by an FAP (32). Although, if the substance produced through this new technology can be shown to be identical to a GRAS substance, it probably would be cleared for use without being labelled as a food additive. Normally an FDA GRAS status is identified with the substance itself and its intended limitations on use, without mention as to its method of manufacture. It appears that the FDA will, however, require clearance by either a food additive regulation or by a regulation affirming GRAS status for products derived through rDNA techniques (33).

A number of the compounds used for flavoring materials are quite volatile, such as acetaldehyde, and are readily recoverable in pure form from existing microbial cultures or their derived enzyme systems. Thus the use of non-GRAS microbial or enzyme systems, within reason, could presumably be used to produce volatile GRAS flavor substances. Although it should be noted that the method of manufacture could be taken into consideration if any question of purity of a GRAS substance produced by a non-GRAS biological system is utilized.

In the systems developed by us described above, attention has been directed at compliance with regulatory considerations, primarily FDA. The production of ethyl acetate and acetaldehyde, both GRAS flavor substances, was accomplished using a GRAS yeast, *C. utilis*. The medium containing basic salts and either glucose or fermentation ethanol should not raise any questions regarding potential toxicities. The end products being very volatile can be readily isolated even if there had been any concern over the microbial system being employed. Additionally, the spent yeast produced in this process is one of only three GRAS yeasts allowed for use in food products destined for human consumption, and can therefore give a by-product credit to this process as discussed earlier. It is tempting to go into nature to isolate new microbial systems but the potential costs can be staggering from a regulatory standpoint. A good example of this is the \$60 million that Rank Hovis McDougall's company in the U.K. spent developing and seeking regulatory approval for a *Fusarium* SCP process (34).

The non-aqueous lipase system for flavor esters developed by our group used components and preparative techniques for enzyme immobilization, that would not only be cost effective and simple but also meet regulatory requirements. The enzyme could have been immobilized by a number of methods however for the intended application only (i) adsorption (ii) ionic bonding or (iii) glutaraldehyde cross-linking would be

appropriate. Adsorption of the lipase to silica gel was chosen as it is an extremely simple method eliminating the need for potentially dangerous chemicals. Strict regulations exist for food-related processes especially where solvents are involved. Hexane was chosen for this process as it is used almost universally for plant material extraction protocols (35). A typical allowable maximum solvent residue in natural extractives is 25 ppm (36). Finally, the lipase from *C. cylindracea* had also had a precedent set for use in food (cheese ripening and butter fat modification).

Industries dealing with food or pharmaceutical products are beginning to incorporate biotechnology into their product development schemes although the routes being taken are rather limited. Large deviation from the use of a select short list of GRAS microorganisms or their derived enzymes is not common practice (8). Where possible 'classic' genetic and/or physiological manipulation of these organisms is done in order to facilitate regulatory compliance and approval. As industry and government regulatory agencies work closer together to clear up the concerns over the use of 'new' biotechnology, including genetic engineering, more applications will emerge. Apart from compliance with government regulations, it is critical that the industry itself set its own high standards as their liabilities are not necessarily protected by government guidelines (12).

INDUSTRIAL INVOLVEMENT IN BIOTECHNOLOGICAL FLAVORS DEVELOPMENT

Unlike the pharmaceutical industry, food processing industries tend to be market driven rather than technology driven (7) and is much more receptive to trends such as the demand for natural flavors. It is anticipated that there will be more investment in biotechnological flavor development especially as the technology proves itself and that existing technology needs can be satisfied by these new approaches.

The majority of large companies' involvement in biotechnology is in the form of R&D contracts to small, entrepreneurial companies. This is a strategic approach which allows for a minimal investment of capital in order to test if the technology developed can be applicable to their needs (36). If the impact is significant, an in-house program would then evolve. A number of companies have taken this approach including: Firminech with DNA Plant Technology for improved production of flavors; W.R. Grace with Synergen for development of microbial systems for flavors; General Foods, although they do have an in-house program, have established links with ESCAGENETICS. F&C International (Canada) Ltd. has established a research collaboration

with the National Research Council, a Canadian Federal Government research organization.

With the projected increase in natural flavors demand expected to grow at an annual rate of about 20% (12) and the current average price of about \$150-200/kg more companies are expected to join in this endeavor. At present only about half a dozen companies are involved in developing natural flavor/aroma chemicals with some retaining a number of their products for captive use in their own flavor blends. It has been estimated (12) that one of the larger companies has upwards of 25-30 natural compounds developed.

FUTURE DIRECTIONS

A number of areas will see significant increases in research activity in the future and include:

- A greater understanding of the capabilities of biocatalysts will allow for an upgrading and increased commercial exploitation of many unutilized biological substances such as certain components of essential oils.
- The trend toward more healthy lifestyles has also encouraged a demand for unsaturated and low fat diets which has increased the use of plant rather than animal products. The preference of 'meaty' flavors will necessitate more use of fermentation-derived flavoring agents such as 5'-nucleotides including inosinate and guanylate, along with hydrolyzed yeast. Overproduction of enzymes to do the hydrolysis, by rDNA techniques, could allow for use of yeast extracts or autolysates directly without the need for purification of RNA by chemical means (20). Recently, a group in Scotland has succeeded in cloning genes for this purpose in yeast which can convert RNA of any origin to these flavor-enhancing compounds (37).
- Potential development of 'industrially robust' enzyme-mimetic systems to allow for specific biotransformations. These mimics would be catalytically active but not be dependent upon the amino acid backbone of existing enzymes.
- An increased use of plant cell culture, for production of plant-unique flavors, will appear as more is understood on underlying control and synthetic mechanisms. Expression of certain plant genes in microorganisms will also be done. An early example of this is the 'taste active' plant protein thaumatin (2500-3000 times sweeter than sucrose) being expressed in microbial systems. For industrial scale production many problems exist with large scale plant cell

cultivation as a result of sensitivity to shear forces (cell breakage), and their tendency to clump thereby limiting mass transfer (nutrient limitation). Enabling technologies such as appropriate bioreactor design and suitable production media must be developed (10).

- Numerous food products will be upgraded to 'value-added' products by the incorporation of flavorings with appropriate functional properties extending beyond flavoring. An important area of research will be in those flavors imparting antioxidant and preservative function (38). Sage and rosemary extracts have been shown to function as antioxidants while those of nutmeg, mace and bay leaf impart preservative effects inhibiting growth of Clostridium botulinum. Microbial or enzyme production of specific flavors with these properties should be possible.
- Certain volatile aroma compounds have been shown to bring about positive changes in mood or general well-being and is encompassed in a new discipline called aroma therapy. It is unlikely that large demands on certain aroma-therapeutic compounds could be satisfied by traditional technologies. Biotechnological approaches could satisfy these requirements for aroma therapeutics.
- The future for production of natural flavors through the use of biotechnological means holds much promise. Researchers and industries interested in this exciting area however, will only be successful if they maintain an awareness of market trends, and of equal importance, knowledge of regulatory requirements.

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Chapter 10

Neurophysiology and Stimulus Chemistry of Mammalian Taste Systems

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Single unit recordings were taken from sensory ganglion cells innervating oral taste buds in the cat, dog, rat, and goat. Neurons were divided into 9 groups largely according to stimulus chemistry. A sodium-lithium system was seen in the rat and goat but not the cat and dog. Amino acid responsive neurons were seen in all species except the goat, with major species differences. Amino acid responsive neurons were also, except for the cat, responsive to sugar. A nucleotide system was seen only in the cat. Acid (Brønsted) responsive neurons were seen in all species, but the cat and dog acid taste systems were different from others. A system responsive to furaneol and other compounds present in fruit was seen only in the dog. A system exclusively responsive to alkaloids was found in rat and goat. Type of taste systems present can to a certain extent be related to species' ecology and dentition.

Flavor chemists typically subdivide the perception of food into three types of sensations: taste, smell and flavor. This latter category almost invariably consists of sensations during consumption. The flavor sensations are considered largely to arise from the stimulation of smell receptors, although research has not demonstrated this to be so. From a biological and physiological point of view, these flavor sensations have little reality. In biology, food odors have been found to have little to do with consumption, being primarily concerned with

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orientation. Consumption is under the control of contact chemoreceptors or taste. Flavor sensations must then consist primarily of taste sensations and/or sensations arising from the simultaneous stimulation of both taste and smell receptors.

In this report the neurophysiology of mammalian taste systems is reviewed with especial attention to stimulus chemistry. The neurophysiology described is primarily that from our laboratory, since we have been among the few neurophysiologists concerned with stimulus chemistry. The animals that have been investigated in detail are the cat, dog, goat and rat. Work on other animals is included where comparisons are viable.

Anatomy and Physiology

Four cranial nerves subserve the sense of taste, three of these (facial, glossopharyngeal and vagus) innervate taste bud systems (Fig. 1) and one (trigeminal) supplies free nerve ending receptors. Both of these types of receptors respond to chemical stimuli. Only the taste bud systems of the facial and glossopharyngeal nerves have been studied in sufficient detail with many food compounds.

The neurophysiological preparation used was metal electrode recordings from the sensory ganglion cell bodies in the geniculate (facial nerve) and petrosal (glossopharyngeal) ganglia of anesthetized animals (Fig 1). This preparation permits long term extracellular recordings from sensory neurons with their peripheral and central extremities intact. Neurophysiological measures taken included spontaneous and evoked activity, and receptive field papillae system mapping with latency measures.

The spike trains recorded from first order taste neurons have some unusual characteristics (Fig. 2). All taste neurons have a certain level of spontaneous activity. This spontaneous activity is often of a highly complex nature. "Bursting", in which the spikes appear in short relatively fixed intervals are common, and "grouping" in which a pseudo-discharge appears is also not unusual. When excited by optimal stimuli two types of discharge may occur. In one, the spikes are tonically occurring with usually a fairly rapid decline in the first few seconds. In the other type, the spikes may appear in groups, often after a long latency. The first type of discharge is common to most geniculate neurons; the second to some geniculate ganglion units and most petrosal ganglion units. Examples of evoked discharges recorded from peripheral sensory ganglion cells are presented in Figure 2.

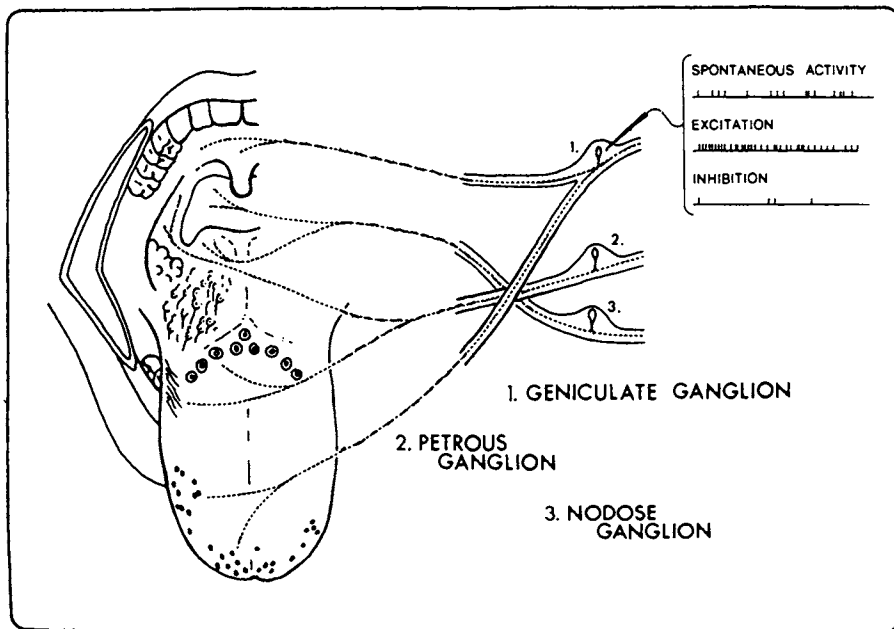
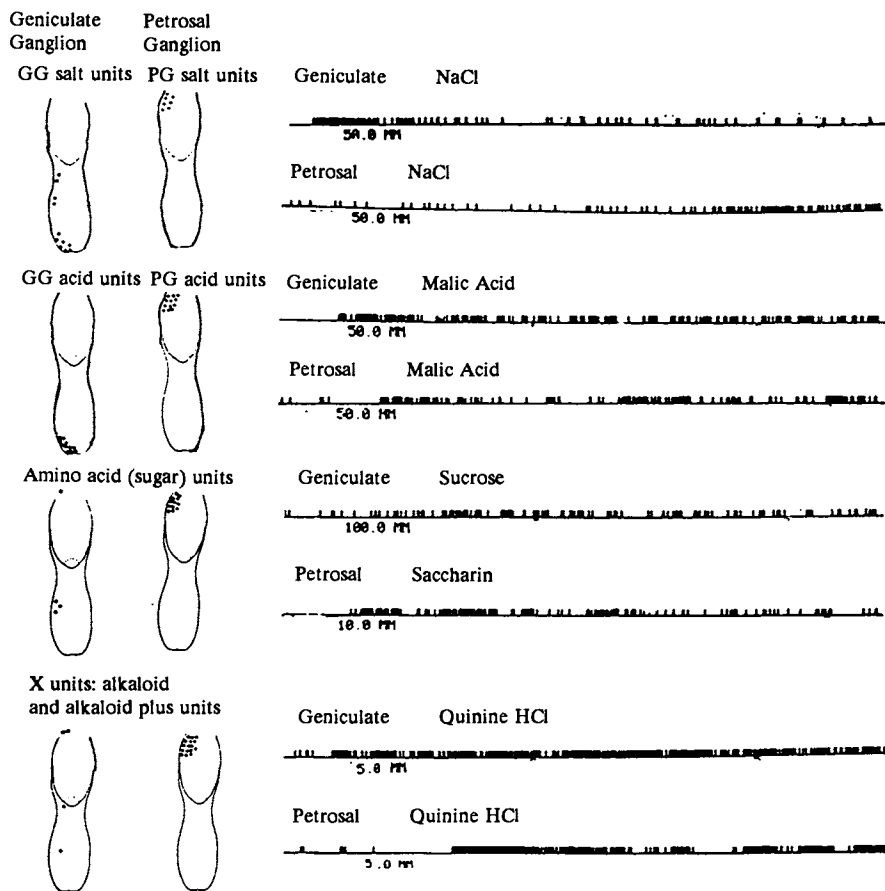


Figure 1. Diagram of the three cranial nerves and associated sensory ganglia that innervate taste buds. As illustrated, electrical recordings were taken from single neurons in the ganglia. Geniculate ganglion in facial nerve; petrosal in glossopharyngeal; nodose in vagus.



DOT PLOT 12 SEC. PER LINE

Figure 2. Taste systems of the rat geniculate (GG) and petrosal (PG) ganglia. Location of receptive fields indicated by a dot on tongue for each neuron studied. Examples are shown of elicited spike discharge for neurons from the six different neural groups identified.

Neural Groups

In every animal studied, the neurons could be divided into a number of neural groups according to their neurophysiological characteristics and the chemical stimuli to which they were responsive. The neural groups that been described in the mammalian geniculate ganglion (GG) and petrosal ganglion (PG) are listed in Table 1, along with some of their characteristics. Geniculate ganglion neurons have been studied in four species (1), but petrosal ganglion units have been studied only in the rat (2). The geniculate ganglion units can be placed into at least seven different neural categories, but a group may be absent from one species or may respond to a somewhat different stimulus array. The neurons in the rat petrosal ganglion have been tentatively divided into four distinct groups, but two of these groups are similar to rat geniculate ganglion groups. All told, at least nine distinct peripheral taste systems can be distinguished in the four species studied. Most of these neural groups have also been distinguished in peripheral fiber recordings in other laboratories (1).

The main criteria used to classify the units in Table I were stimulus response measures; i.e., the units discharged or were inhibited by different chemical compounds. In addition, other criteria were used to supplement the chemical stimulus response differentiation. Thus, the two main groups in the cat (acid units and amino acid units) can also be differentiated by spontaneous activity measures, latency to electrical stimulation, area of tongue innervated, and differential response to solution temperature (3-5). This comparative work has led to a modular view of peripheral taste systems in which the different neural groups are seen to have distinct receptors responding to distinct types of chemical signals (e.g., Brønsted acids and Brønsted bases), with either excitation or inhibition. The stimulus chemistry of these groups will be briefly described.

Salt Responsive Units. One of the neural groups with the simplest stimulus chemistry is the GG salt system found only in the geniculate ganglion of the rat and goat. These units are only responsive to sodium or lithium salts. When a series of Cl salts with different cations are examined, only those with Na and Li elicit large responses (Fig. 3). Na and Li are effective with other anions as well, although responses are largest with I and F (6).

The only other group of neurons responsive exclusively to salts was the rat PG salt unit group (Fig. 3). These units of the petrosal ganglion responded to a variety of Cl salts, not showing the Na,

Table I
Mammalian Peripheral Neural Taste Groups

Geniculate Ganglion (Facial Nerve): GG. Petrosal Ganglion
(Glossopharyngeal Nerve): PG

Group	Species	Stimuli
1. GG Salt System	Rat and Goat only	Na ⁺ and Li ⁺
2. GG Acid System	Rat and Goat different from Cat and Dog	Brønsted acids
3. GG Amino Acid System	Cat and Dog	Proline, Cysteine, Hydroxyproline, Lysine, Alanine
4. GG Nucleotide System	Cat only	ITP, ATP, etc.
5. GG Furaneol System (Probably mainly PG)	Dog only	Furaneol, Ethyl Maltol, Methyl Maltol
6. PG Amino Acid System (also in GG)	Rat	Sugar, Saccharin, Amino Acids
7. PG Alkaloid System (also in GG)	Rat and Goat	Atropine
8. PG Acid System	Rat	Restricted set of carboxylic acids
9. PG Salt System	Rat	KCl, CaCl ₂ , MgCl ₂ , NaCl

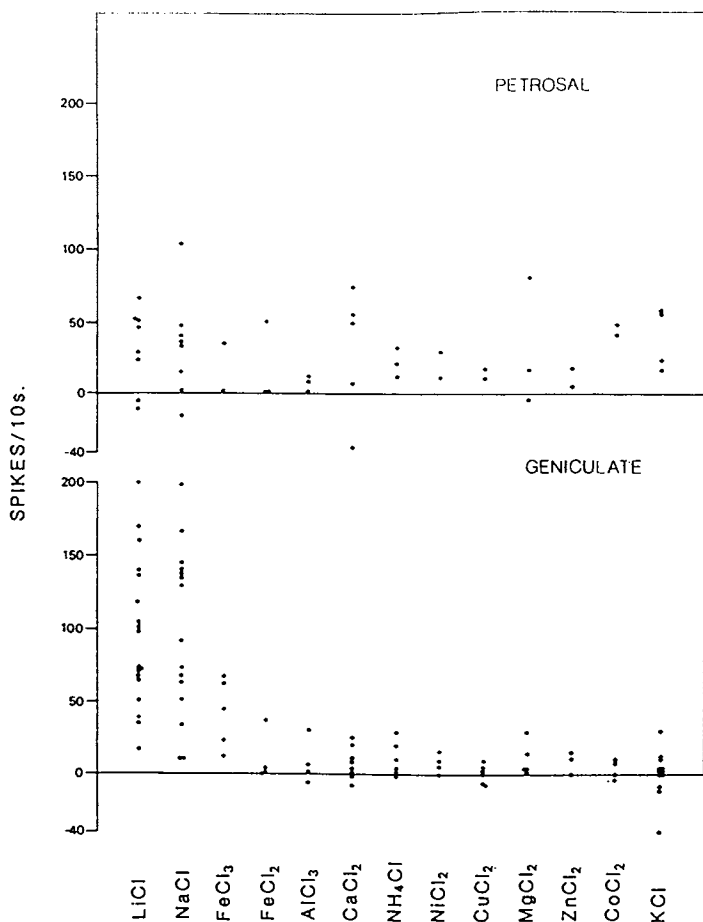


Figure 3. Responses of rat GG salt units and rat PG salt units to a series of chloride salts. Each point represents the spike response in a 10 second period to a 50mM solution. Note the exclusivity of response of GG salt units to NaCl and LiCl.

Li exclusivity shown by GG salt units. In addition, they exhibited low discharge rates and sluggish evoked discharge patterns.

Nucleotide Responsive Units. Certain of the cat units were observed to discharge only to nucleotides and other phosphate compounds. In addition to these nucleotide units, some other cat units also discharged to nucleotides and other substances. In general, the di- and tri-phosphate nucleotides were the most stimulating for the nucleotide units although both AMP and IMP elicited respectable responses. Tetrasodium pyrophosphate was a strong stimulus and sodium phosphate a moderate one. These units exhibited long latencies to electrical stimulation, low spontaneous activity rates, and "grouping" evoked discharge patterns. No specific regional distribution of receptive fields was observed.

Furaneol Responsive Units. Found only in the dog were a small number of units responsive to a variety of plant compounds known to be intensely sweet for the human. Especially active was the compound furaneol [2,5 dimethyl-4-hydroxy-3(2H) furanone] and the closely related ethyl and methyl maltol. Also stimulating were ammonium glycyrrhizinate and (slightly) neohesperidin dihydrochalcone. Some units were also responsive to quinine. No response was shown to either amino acids or sugars, nor were salts or acids stimulating. These furaneol units were the only units in any species responsive to intense sweeteners. Furaneol and other compounds were tested on many cat units and some rat units but no discharge was evoked. The dog units possessed small fibers and displayed "grouping" discharges, often with long latencies. It is quite likely that these units are representative of a larger population of neurons in the petrosal ganglion of the glossopharyngeal nerve, a preparation not studied in the dog.

Acid Responsive Units. All species possessed an acid taste system although this system was not identical from species to species. The system was labeled "acid" because the most stimulating compounds were Brønsted acids and the least stimulating were Brønsted bases. The most excitatory compounds were carboxylic acids for all species. Also stimulating, but at a variable rate, were phosphoric acids and a small number of nitrogen compounds functioning as Brønsted acids. Histidine, functioning as a Brønsted acid, was active in all species. The compounds with phosphoric acid groups were least active on the rat and goat. Salts such as NaCl and KCl were active on the rat and goat though less so.

The acid units in the cat were studied in the most

detail. It was found that imidazole was even more stimulating than histidine. A small group of nitrogen heterocycles when protonated, were the most excitatory compounds for the cat. The heterocycles, imidazole, thiazolidine, and pyridine with their relatively high pK's were extremely exciting at a pH of 7.0. In the cat's normal diet of meat, a pH below 5.5 is rarely encountered, rendering most carboxylic and phosphoric acids nonstimulating. Present in large quantity in animal tissues in free form however are histidine dipeptides: anserine, carnosine and ophidine, depending on animal species. Dog acid units were almost identical to those in the cat.

Present in the rat petrosal ganglion was another set of acid units responsive primarily to certain carboxylic acids. Unlike the cat (7), the rat was unresponsive to some carboxylic acids even though they were in low pH solutions (2). Possibly the same is true for the rat GG acid units which were not investigated in as much detail. PG acid units, unlike all other acid unit groups, responded in a "grouping" discharge fashion. Goat acid units seemed in between carnivore and rat acid units, being more responsive to phosphate compounds.

Alkaloid Responsive Units. Present in the rat and in the goat were units which were responsive primarily to a small group of alkaloids. These units were found in the geniculate ganglion where they were few and innervated the back part of the tongue. They were found in larger number in the rat petrosal ganglion. These units exhibited long latencies to electrical stimulation, indicating small fiber diameters and displayed "grouping" evoked discharge patterns. The rat alkaloid units were maximally discharged to atropine, quinine, colchicine and sparteine. The goat units were maximally discharged by pilocarpine, quinine and colchicine. Few other non-alkaloids were active although CaCl_2 was stimulatory for some rat and goat units. A few units in the cat were maximally discharged by alkaloids (mainly quinine and brucine) but they were not studied with an array of alkaloids.

Amino Acid Responsive Units. Found in the geniculate ganglion of the cat, dog, and rat, but not in the goat, are neural groups highly responsive to amino acids (Fig. 4). The amino acid units of the dog and rat, but not of the cat, are also responsive to sugars. The amino acid units of all three species are also responsive to nucleotides but less so in the rat. The rat amino acid units are distinct from those in the carnivore in that different amino acids are maximally stimulatory, and the discharge rates are usually much lower. An amino acid group of neurons was also detected in the rat petrosal

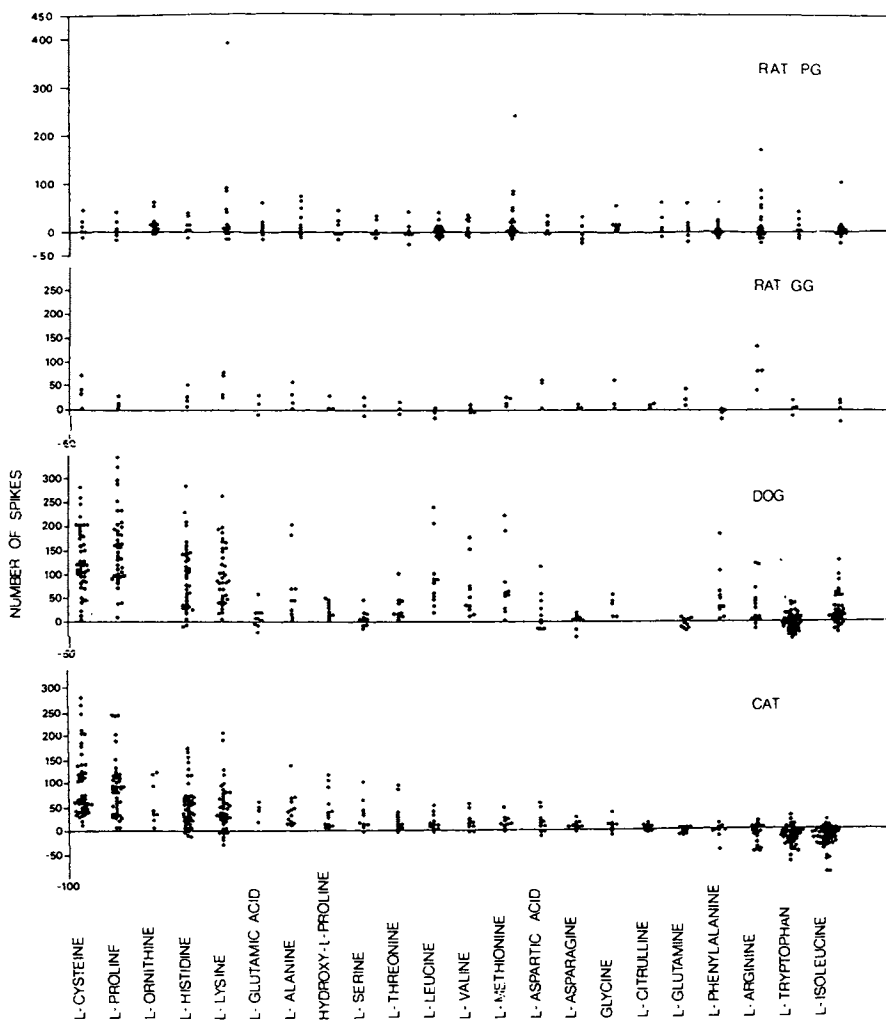


Figure 4. Responses of the amino acid groups from three different species. All solutions 50 mM. No amino acid units were seen in the goat.

ganglion where, unlike in the geniculate ganglion, these units formed the most populous group. The rat geniculate ganglion amino acid units are similar in general in their stimulus response properties to the rat petrosal ganglion amino acid units.

Cat amino acid units are essentially discharged by two distinct types of compounds: those containing phosphate groups, such as sodium phosphate, tetrasodium pyrophosphate, and all di- and triphosphate nucleotides; and certain amino acids. Monophosphate nucleotides elicited little response from amino acid units. The most effective amino acids in eliciting discharge were L-proline, L-cysteine, L-ornithine, L-lysine, L-histidine, and L-alanine. Certain amino acids such as L-tryptophan, L-isoleucine, L-leucine, L-arginine, and L-phenylalanine tended to inhibit cat amino acid units. The inhibitory property of the L-amino acids has been related to the hydrophobicity of their side chains

Cat amino acid units have been studied with a variety of stimulus solutions including natural foods such as chicken and liver (8, 9). The most excitatory compounds, as indicated above, tended to be either compounds with a phosphate group or compounds with a nitrogen group. The D amino acids tend to be less stimulatory than the L forms. The most effective stimuli found included small heterocyclic nitrogen compounds such as pyrrolidine. Inhibitory compounds were mostly alkaloids, nucleotide bases and certain heterocyclic nitrogen compounds. The response of cat amino acid units to nitrogen heterocycles could be related to two structural and chemical factors: (i) a steric factor (in particular ring size) and (ii) the relative basicity of the heterocycles as indicated by pKa values. Cat amino acid units were also discharged by NaCl and KCl solutions, but the thresholds were above 50 mM.

Although the most stimulatory amino acids were identical in the dog (e.g., L-cysteine, L-proline, L-lysine, L-histidine and L-alanine), interspecies differences could be related to the side chain properties of the amino acids. Thus, amino acids with hydrophobic side chains were normally inactive or inhibitory in the cat, but were often excitatory in the dog. Conversely, amino acids with acidic side chains tended to be somewhat more excitatory in the cat.

The response of the rat amino acid units to amino acids was quite distinct from that of the cat and dog. Little response, for instance, was elicited from rat amino acid units by most of the di- and triphosphate nucleotides, and sodium phosphate was inhibitory. The most effective amino acid for rat units was L-arginine, a compound inhibitory in the cat and a minor stimulus in the dog, followed by L-lysine and L-aspartic acid. L-

proline was largely inactive in the rat. Few of the rat amino acid units discharged at high rates. Rat units were also responsive to sugars and saccharin.

Summary of Mammalian Neural Groups

The different neural groups distinguished in the geniculate and petrosal ganglia are summarized with respect to species in Table II. The animals studied in the geniculate ganglion have been supplemented with three species studied only in the chorda tympani: the hamster (10, 11), the squirrel monkey (12) and the macaque (13). The amino acid units in the two primates seem to represent the two different types of amino acid units seen in the ganglion preparation. The squirrel monkey amino acid units seem quite similar to dog amino acid units even though the investigators themselves classify them as salt units. The macaque units on the other hand display the unusual grouping discharge patterns shown by rat amino acid units. The human is included in this table because the different human sensations seem to represent psychophysical signs of excitation or inhibition of different neural groups (14). On the basis of chemicals active, the human acid units seem more like those of the cat and dog than the rat or goat (7, 14). The human sodium system seems identical to that in the rat, hamster and goat (15). The human clearly possesses a facial nerve amino acid system similar to the carnivore (16) and a petrosal system similar to the dog furaneol system (14, 17). The human also possesses a glutamate system, yet undetected in any experimental mammal (18, 19).

Discussion

The modular taste systems summarized for mammals in Table II are quite similar to the modular taste systems that have been observed for invertebrates, such as lobsters and crayfish (20, 21). The most extensive invertebrate taste research has been performed on caterpillars (22, 23). In 20 different species of caterpillars, 12 different neural groups were distinguished.

Viewed in terms of neural groups, the experimental animals detailed here constitute a diverse group of organisms. The rat and the hamster seem to possess identical geniculate ganglion systems. Should the rat and hamster prove to be representative of rodents in general, this sodium, acid, amino acid-sugar taste system may be common to most or all rodents (of which there are around 2400 species). The rodent taste system is also quite similar to that of the goat; although no amino acid-sugar system has yet been detected in the

Table II
Summary of Mammalian Peripheral Neural Taste Groups
(See Text)

Neural Groups	Species							
	Cat	Dog	Rat	Goat	Ham-ster	Sq. Monk	Mac. Monk	Man
<u>Facial</u>								
GG Salt (Sodium)			X	X	X		X	X Salty
Amino Acid Cat type	X	X				X		X Sweet1
Acid, Cat Type	X	X						X Sour
Acid, Rat Type			X	X	X			
Nucleotide	X							
<u>Glossoph.</u>								
Amino Acid, Rat Type			X ^a		X ^a		X ^a (?)	
Furaneol		X ^a						X ^a sweet2
PG acid			X					
PG salt			X					
Alkaloid	?		X ^a	X ^a				
Glutamate ^b								X ^b umami

a: Also in facial

b: Psychophysics only

goat, the alkaloid system, sodium system and GG acid system of the goat are like those of the rat. Perhaps the taste systems of many mammals capable of living on plant foods contain basic similarities.

The cat and the dog, on the other hand, possess taste systems that have little in common with rodents and goats. Not only do they have no sodium system, but their acid and amino acid systems are also markedly distinct. Although the cat and the dog have two systems, the acid and amino acid systems, in common, both also possess a taste system which the other does not: the cat a nucleotide system and dog a furaneol system.

The primates have been inadequately studied, but those two with adequate single unit data suggest that the organization of primate taste systems is no simple matter. It is not obvious for instance, why the squirrel monkey may have an amino acid system like a carnivore and the macaque one like a rodent. The human taste system further complicates matters since man can best be viewed as a composite, having a sodium system like the rat and goat, carnivore acid and amino acid systems, a furaneol system like the dog and a glutamate system unlike any other mammal studied (14).

The compounds active on both vertebrate and invertebrate taste systems constitute a select group of low molecular weight compounds. The compounds include organic acids, salts, nucleotides, amino acids and a variety of secondary compounds, notably alkaloids but also others, including here furaneol and ethyl and methyl maltol. Just why certain of these compounds are active on taste systems is often a moot point. The significance of none of the acid systems, for instance, is obvious from an ecological standpoint, nor is it apparent why certain acids are so potent. It is also not clear why the two amino acid systems are so distinct, nor why proline and cysteine should assume such a large role in the carnivore taste system.

The taste systems which are ecologically obvious, however, are the GG sodium system and the dog furaneol system. The sodium system is not present in carnivores but is present in herbivores and omnivores. The importance of this system in the rat and goat cannot be overemphasized since half of the taste neurons in the geniculate ganglion are devoted to sodium sensing. The presence of a sodium system in animals that may subsist entirely on plant substances is quite obvious since Na is often present in minuscule quantities in most plants (24). Both the rat and goat exhibit a salt hunger and can with saline solutions regulate their sodium intake to supply their sodium need. Although the dog (and related canines) may subsist for fairly long periods of

time on fruit or other plant substances, it cannot regulate its sodium intake by taste (25).

The dog units were labeled furaneol units because this compound is found in large quantity in many fruits (26). Besides being intensely sweet, this compound also has a fragrant odor and is a character impact compound for many fruits. It is believed that this dog furaneol taste system is specific for fruit and is linked with the seed dispersing function of the dog. The presence of this taste system and its absence is readily detectable in the natural eating behavior of canines and felids. In a natural environment canines will supplement their small animal diet with fruit of the season, unlike felids. Nucleotide responsive units are relatively rare in taste systems. The only other vertebrate nucleotide taste system that has been described is in the puffer fish (27). This fish facial nerve taste system, like that in the cat, also responded to a wide variety of nucleotides and to inorganic phosphate compounds. In invertebrates, nucleotide taste systems have been described for blood sucking animals where they are common (28).

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Chapter 11

Temporal Aspects of Flavoring

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The aroma of a food product is often measured by a sensory technique called descriptive profiling, in which flavour experiences are described by a panelist as a set of component impressions or sensations of varying degrees (1). Profiles do not hold explicit information about the temporal characteristics of a flavour, its persistence and the times of appearance of the individual notes. Nevertheless, the rank order of the attributes sometimes reflects, to some extent, the order of appearance of the corresponding impressions. Some notes are then said to be released "early", others "late".

However, there is little published experimental evidence that demonstrates a relationship between the temporal features of aroma perception and the stimulus concentration near the sensory receptors. In the following we describe some experiments that examine the issue directly and some theoretical ideas that appear to explain the results.

Psychophysical Measurement

When carrying out psychophysical measurements on a flavoured food we usually define the system as:

$$\text{flavour/matrix} > \text{sensory response}$$

where the flavour/matrix is e.g. diacetyl in margarine and the sensory response is a magnitude score representing perceived intensity.

This view, however, is too simple. In reality we have to consider a stimulus-response system where the stimulus is defined as a concentration, not in the product but at the receptor sites and not as a single value but as a function of time. Likewise, the response should be measured as a function of time.

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The system can then be formulated as follows:

flavour/matrix > stimulus (t) [psychophysical function (t)] >
response (t)

To be able to understand this system we have developed:

- a methodology to measure the concentration of flavour as released from a matrix, at the nose, breath-by-breath;
- a time-dependent form of the psychophysical function relating stimulus to perceived intensity;
- an improved methodology to measure perceived intensity as a function of time (I/t).

Mass-spectrometric breath-by-breath analysis

Breath-by-breath analysis of gases and volatiles is well known in medicine (1). The experimental techniques used, however, were not very well suited to our needs. For our purpose we needed a simple, reliable inlet system without extensive filtering and pressure reduction, but with a high sensitivity and short response times.

Therefore a (semi-) continuous measuring methodology, like MS was considered. Trace analysis by MS via a membrane separator was known (2), but the decay times of the signal precluded breath-by-breath analysis.

When the construction of the separator was studied more closely, it appeared that the device traded response time for sensitivity. A fast response requires a small internal volume, but a high sensitivity requires a large membrane surface. Reducing the surface area and the internal volume resulted in very short response times and sufficient sensitivity (see Fig. 1).

The setup responsible for these improvements is depicted in Fig. 2.

Via two small glass pipes, one in each nostril, a small pump sucks 550 ml/min of air from the nose and past the membrane. The MS takes 20 data points/s and the result is a full breath-by-breath quantification of volatiles released from the mouth during mastication.

A typical result is shown in Fig. 3a.

One can see a very sharp leading edge, followed by an exponential decay as the flavour is depleted from the oil. To obtain panel results, the individual curves are modelled by fitting a function consisting of two exponentials, one representing the rise and the other the decay of the signal, to the data. This procedure transforms the individual breath-by-breath results into a smooth stimulus curve, characterised by the parameter values of the exponentials (see Fig. 3b).

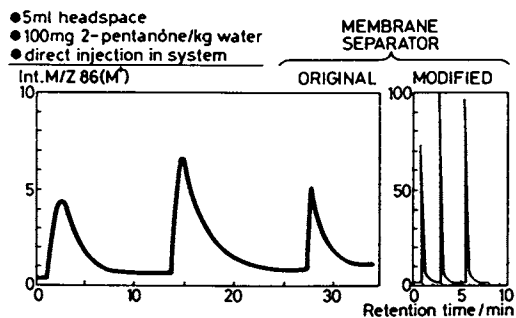


Fig. 1 Peak shape of headspace of 2-pentanone solution in water

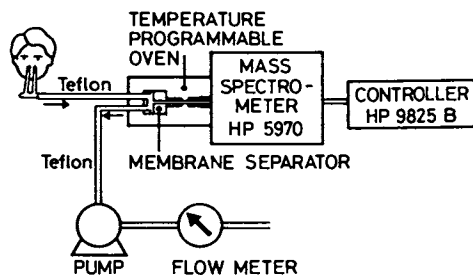


Fig. 2 Lay-out of breath analyzer

The parameter values are subsequently averaged to produce a panel stimulus curve. Apparently, the pentanone is depleted from the liquid layer in the mouth very rapidly. It should be borne in mind that this is not only due to release into the gas phase, but must also be ascribed to uptake into the mouth and upper airways (3).

Fig. 4 depicts the panel curves of the simultaneous release of butanone-2 and pentanone-2 from water. As pentanone-2 is more hydrophobic it is released faster.

Both curves peak at the same time but pentanone-2 peaks higher and is subsequently depleted faster. After about 50 s the release curves cross.

If the flavour characters of these substances would have been sufficiently different the panel would probably have commented that butanone-2 released "late".

A time-dependent form of the psychophysical function

In order to be able to predict the effect of alterations to the time course of stimulation on perceived intensity over time, the static psychophysical function had to be extended.

In the following section, taste and smell will be treated equally. In detail this is not correct but for the line of thought to be developed here the treatment is the same.

We started from Stevens' law (4) including the threshold correction:

$$I = k (S - S_0)^n$$

where

I = perceived intensity as expressed

S = physical stimulus strength

S₀ = unadapted threshold level

k and n are constants

If prolonged stimulation, of any temporal form, is to have an effect on this relationship, i.e. if I becomes I(t), then at least one of the other parameters must also become a function of time. The only well documented effect of prolonged stimulation on the characteristics of taste and smell is adaptation.

Fig. 5a shows the effects of adaptation to a constant stimulus prior to magnitude estimation as measured by Cain (5); the curves relating Intensity to Stimulus strength drop off near the concentration level of the adapting stimulus. At higher stimulus levels, however, they seem to converge.

In Fig. 5b the adapting levels have been deducted from the actual stimulation and results show straight lines for perceived intensity against stimulus minus adapting level. Within Stevens' equation, therefore, it appears that it is the threshold term that is affected by stimulation. We had to find out how, however.

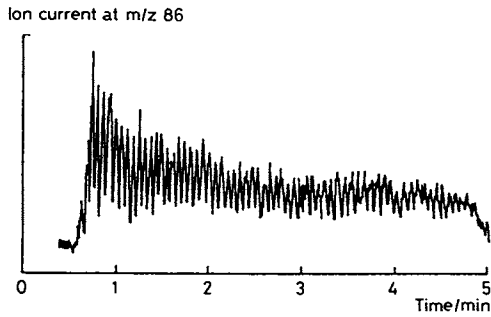


Fig. 3a Breath analysis
100 mg 2-pentatone/kg MCT oil in the mouth

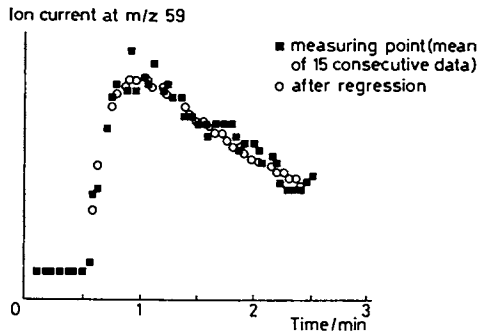


Fig. 3b Single release curve of butanol-2 from water after smoothing and after regression. Each black square represents the mean of 15 consecutive data points in a single experiment. The open circles represent the best fitting curve.

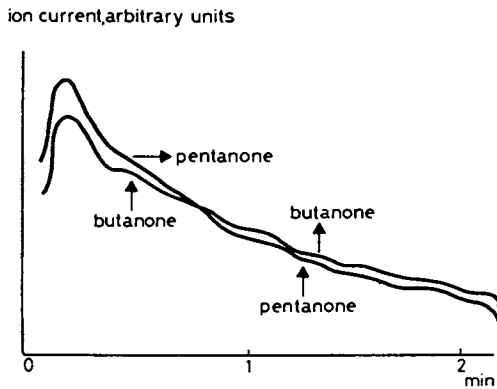


Fig. 4 Simultaneous release of butanone-2 and pentanone-2 from water

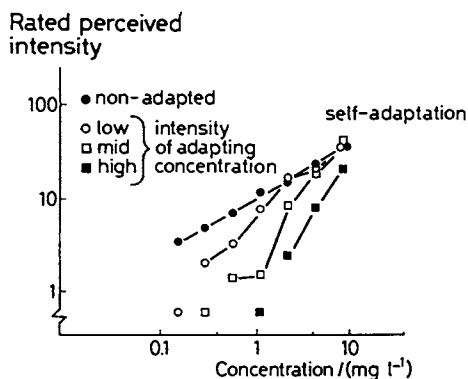


Fig. 5a Perceived intensity (I) vs stimulus concentration of pentanol in an olfactometer experiment, under various conditions of pre-adaptation (after W.S. Cain, *Percept. Psychophys.* 7 (1970) 271)

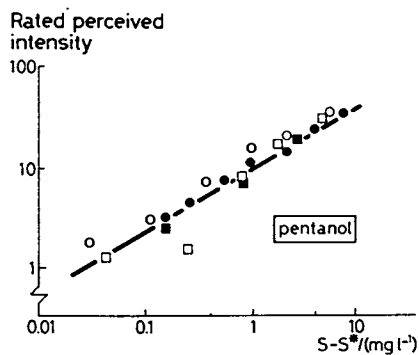


Fig. 5b Perceived intensity (I) of pentanol vs stimulus concentration (data from Fig. 5a) replotted after subtraction of the adapting concentration from the stimulus concentration (I vs. $S-S^*$)

In Fig. 6a data by Hahn (6) are shown. Hahn determined the effect of three levels of salt concentration, as a function of time, on the threshold of perception.

If we now define two extra parameters:

S^* = threshold level as a function of time
 A = adaptation constant

the conclusions drawn from these data can be used to construct a differential equation relating threshold level to stimulation. The conclusions from the measurements by Cain and Hahn are:

- After prolonged stimulation the threshold rises to a level which lies above the level of stimulation, the difference being roughly equal to the original unadapted threshold level (for $t \rightarrow \infty$, $S^* \rightarrow S + S_0^*$).
- The adaptation proceeds faster when the difference between threshold and

stimulus is bigger; $\frac{dS^*}{dt} \propto S - S^*$

- The time it takes for the threshold to reach the stimulus level is longer

for a stronger stimulus; $\frac{dS^*}{dt} \propto \frac{A}{S}$

Putting these conclusions together, we arrive at:

$$\frac{dS^*}{dt} = \frac{A}{S} (S_0^* + S - S^*)$$

which can be integrated to give

$$S^* = S_0^* + e^{-\int \frac{A}{S} dt} \cdot A \int e^{\int \frac{A}{S} dt} dt$$

in case of constant stimulation this reduces to

$$S^* = S_0^* + S (1 - e^{-\frac{At}{S}})$$

Fig. 6b shows the best fit of this equation to Hahn's data. Assuming that this relationship would also be valid for non-constant stimulation, we can try to predict what would happen if we would use a stimulus like the one we measured with the MS/breath method, after smoothing, at two levels of concentration (See Fig. 7).

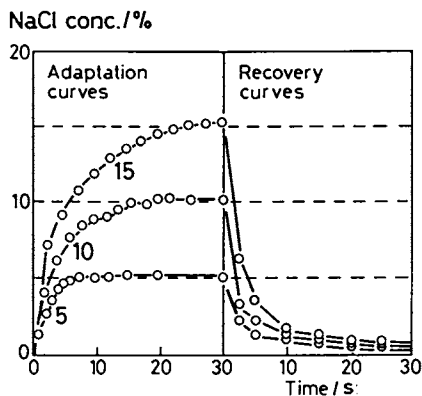


Fig. 6a Perception of thresholds vs. time under stimulation of 5, 10 and 15% sodium chloride solutions [after H. Hahn, Z. Sinnephysiol. 65 (1934) 105]

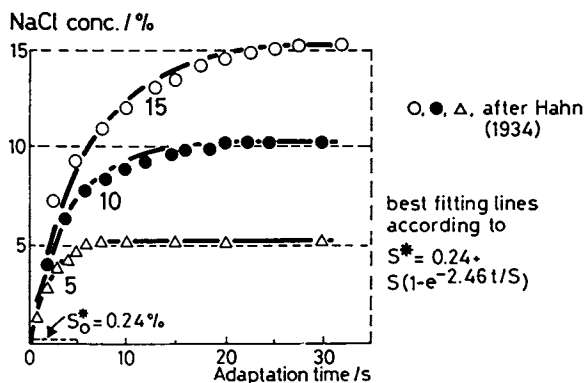


Fig. 6b Perception thresholds vs. time under stimulation of 5, 10 and 15% sodium chloride solutions

The theory predicts that in both cases the threshold level rises linearly with stimulation in the first part of the curve and keeps rising until the threshold level equals the level of stimulation. Since we still use Stevens' law which has now taken a time-dependent form: $I = k(S-S^*)^n$, we may predict that the higher concentration will be perceived

- more intensely
- at the same time of maximum intensity
- for a longer duration

Now that we have measured the actual stimulus shape and have predicted its perceptual result, we evidently have to measure perceived intensity as a function of time.

Measuring perceived intensity over time (I/t)

Methods for scoring perceived intensity over time are known in the literature (7). They make use of a pen recorder, a dial potentiometer or a "mouse" device coupled to a personal computer. The panellists move the pen or dial up when perceived intensity increases and down when it drops off. The data are pooled by calculating mean Intensity values.

The procedure contrasts with the above described MS/breath data pooling method. In both cases we start with individual I/t curves. In the MS/breath case these are parametrized, so that after pooling the parameter values of the panel curve are the mean values of the individual parameters.

The literature method for perceived intensity over time does not produce such panel curves.

Du Bois and Lee (8) describe a method which does produce panel averages for the three main parameters: maximum perceived intensity (I_{max}) as scored by the individual panellists, the time at which this occurs (t_{max}) and the extinction time (t_{end}). Since these parameters do not produce a complete curve, we have developed a method which produces complete curves which can be considered to be real panel averages. This method is carried out as follows:

All individual curves are normalised in the Intensity direction by calculating the geometric mean of all individual I_{max} values and multiplying each

individual curve by $\frac{I_{max}}{I_{i,max}}$ (geom)

Subsequently all half curves before and after $t_{i,max}$ are averaged in the time direction. Again the geometric mean is taken because a check on the distribution of $t_{i,max}$ and t_{i-end} values (ATCS in Fig. 8) showed a log normal distribution. The resulting curve can be considered to be a real panel average.

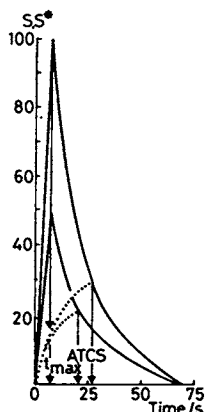


Fig. 7 Dependence of threshold of perception S^* (•••) on an arbitrary time course of stimulation (—) at two levels

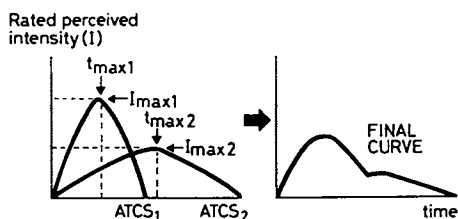


Fig. 8a Schematic representation of the existing procedure. The final curve is obtained after averaging the individual curves in the intensity direction only

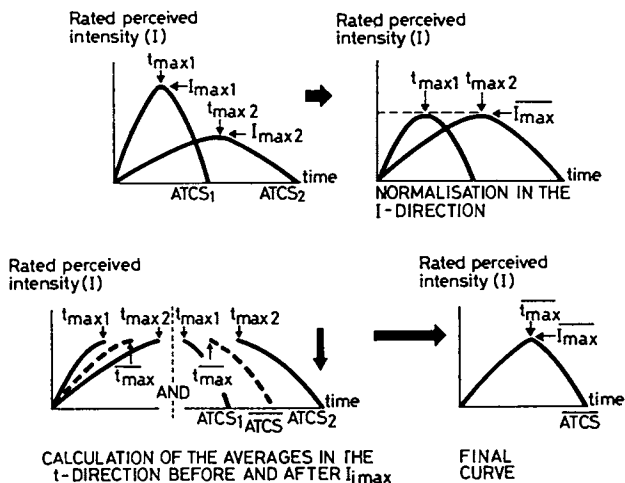


Fig. 8b Schematic representation of the new procedure. The final curve is obtained after averaging the individual curves in both the intensity and the time direction

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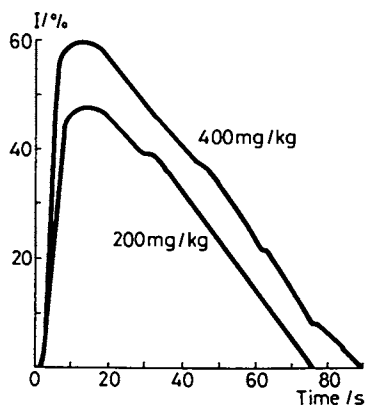


Fig. 9 Perceived intensity (I) vs. time of 2-pentanone in vegetable oil using the slide potentiometer method

Figs. 8a/b show a slightly simplified version of both types of data treatment mentioned. Our approach may be illustrated through the combination of two experiments.

The first involves I/t measurements of two concentrations of pentanonin in vegetable oil. The predicted results are obtained: a higher maximum and at the same time a longer duration for the higher concentration (see Fig. 9).

When these results are compared with those of the measurement of the real stimulus (Fig. 3a) the adaptation effect is evident; perception already ends when the actual stimulus has dropped only to around half of its highest value.

Summing up, we have defined our system as follows:

flavour/matrix > stimulus (t) (psychophysical function (t) > response (t)

We have measured both time-dependent variables:

the actual stimulus and the response, and it has been shown that a suitable time-dependent version of Stevens' law could be constructed from material available in the literature (9, 10).

For stimuli containing more than one component it was shown that different physical release rates, starting at the same time, could very well give rise to perceived differences in release times.

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Chapter 12

Enantioselectivity in Odor Perception

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The first molecular event in odor perception is an interaction of an odorant with a receptor. Evidence exists that these receptors are proteins, i.e. chiral, so this first interaction should be enantioselective, meaning that these receptors react differently with the two enantiomeric forms of a chiral odorant leading to differences in odor strength and quality. In many cases, this fact has been observed. This paper describes the enantioselective syntheses of some known odorants of multiple chemical classes and discusses the differences of the organoleptic properties of their enantiomeric forms.

The mechanism of odor perception is very complicated and the least understood of all our senses. It is well accepted that the perception of an odor, meaning the actual recognition by the brain, goes through a cascade of events.

STIMULUS → RECEPTOR → TRANSDUCTION → PROCESSING

Of all these different steps, the very first one, namely the interaction of a stimulus, i.e. molecules that "have a smell", with the actual receptor is not at all understood. These receptors are supposed to be located in the membrane of the cilia cells, because these cilia are the furthest out of the antennae of the olfactory system, and they have been shown to be excitable by chemical stimuli.

In analogy to other—better understood—receptor systems like some hormone and opiate receptors it is generally accepted that the olfactory receptors are proteins, and there are some facts known that support this hypothesis. One of these arguments is that, sometimes, slight modification of the chemical structure of a stimulus molecule can lead to big changes in the odor impression; this might be qualitative or quantitative.

Proteins are chiral, so they should interact differently with the two enantiomeric forms of a chiral molecule, which should eventually translate into a difference of the odor impression of these mirror images of the molecules. A more detailed knowledge of the relations between the chemical structure of a molecule, including its absolute configuration, and its odor properties will contribute to the elucidation of the receptor mechanism.

Actually there are many examples known where the two enantiomeric forms of chiral compounds have different odors. Table I shows some of them without being exhaustive.

Enantioselective synthesis have become very fashionable in preparative chemistry, and a considerable effort is devoted to their methodology.

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Table 1
Enantiomeric Forms of Chiral Compounds and Their Odors



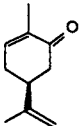
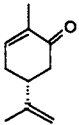
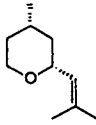
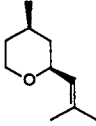
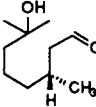
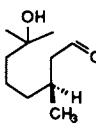
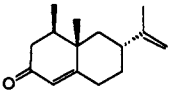
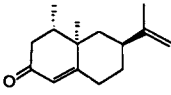
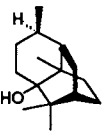
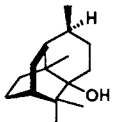
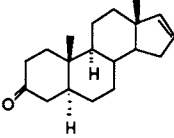
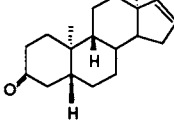
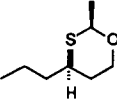
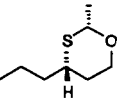
Compound	Odor impression	Lit.
 (+)-Linalool	sweet, petitgrain	[1]
 (-)-Linalool	lavender notes, Ho oil, woody	
 (+)-Carvone	caraway	[2]
 (-)-Carvone	spearmint	
 (+) -cis-Rose oxide	sweet	[3]
 (-) -cis-Rose oxide	powerful, fruity	
 (+)-Hydroxycitronellal	sweet, powerful	[4]
 (-)-Hydroxycitronellal	minty	

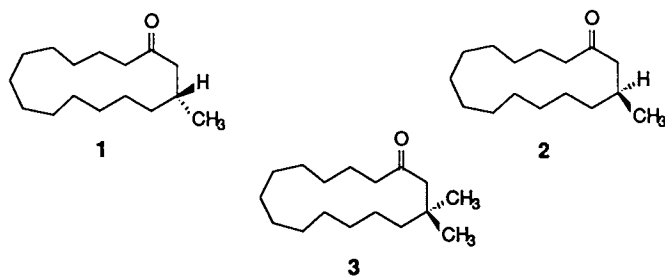
Table 1. *Continued*

Compound	Odor impression	Lit.
 (+)-Nootkatone	t = 0.8 ppm grapefruit, strong	[5]
 (-)-Nootkatone	t = 600 ppm very weak, no grapefruit	
 (-)-Patchouliol	natural patchouli, earthy, cellary	[6]
 (+)-Patchouliol	weak, not reminiscent of patchouli	
 (-)-Androstenone	sweaty, urine musky, strong	[7]
 (+)-Androstenone	odorless	[8]
 (+)- <i>cis</i>-2-methyl-4-propyl-1,3-oxathiane	t = 2 ppb sulfury, rubbery, tropical fruit	[9]
 (-)- <i>cis</i>-2-methyl-4-propyl-1,3-oxathiane	t = 4 ppb flat, estery, camphoracious	[10]

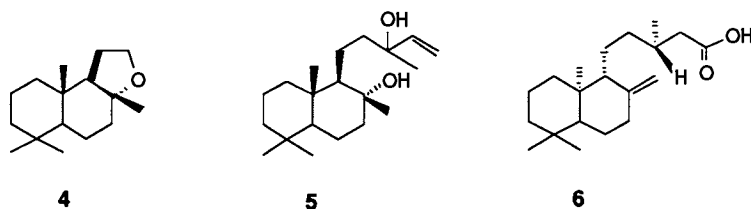
In our continuous interest in the relation of molecular structure and organoleptic activity, we synthesized the enantiomers of some well-known aroma chemicals, and evaluated their odor. For the preparation different synthetic approaches were used, i.e.

- starting with the same material employing reagents of opposite chirality that can either be recovered after use or are lost during the synthesis;
- starting with natural products of known antipodal configuration.

Muscone **1** was discovered in 1906 [11]; its structure [12] and absolute configuration [13] were determined later to be (*R*)-3-methylcyclopentadecanone.



It is, in its racemic form, a highly appreciated ingredient in fine perfumery. Because of its value a number of syntheses have been described [14]. Enantioselective syntheses of the (-)-(*R*) **1** and the (+)-(*S*)-form **2** have been developed [15], however no olfactive description of the two compounds could be found. Following the synthesis of *Nelson* and *Mash*, both enantiomeric forms of muscone were prepared. The optical purity, determined by 360 MHz NMR, using Pr (hfc)₃ as chiral shift agent was 95.5% for the (-)-(*R*) and 97.7% for the (+)-(*S*)-form. The two products show distinct differences in their odor. The natural **1** is described by a panel of perfumers as "very nice musky note, rich and powerful", whereas **2** is "poor and less strong". Thresholds, determined in water, using Guadagni's procedure [16], with a panel of 18 - 20 members, show values of 61 and 233 ppb respectively, giving a calculated threshold of 97 ppb for the racemic mixture, in good accordance with the experimental value of 103 ppb.

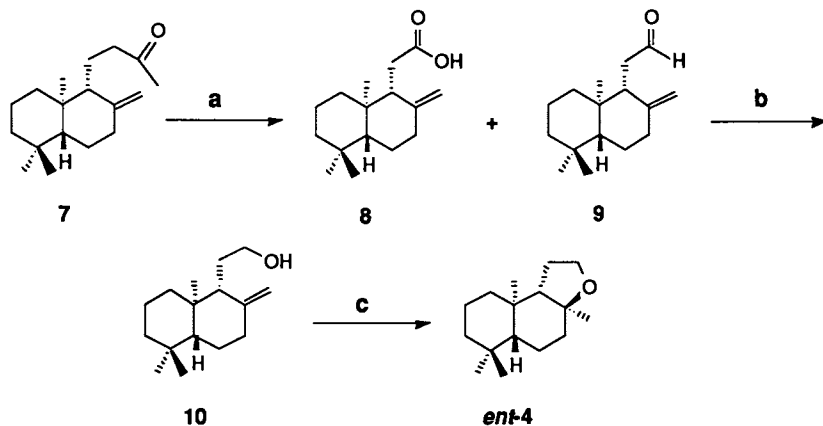


From these results, one could deduce that the methyl group in **2** somehow hinders easy access of the molecule to its receptor. This hypothesis is supported by the fact that 3,3-dimethylcyclopentadecanone **3** is nearly odorless.

The tricyclic ether AMBROX **4**, first synthesized in 1950 [17], was later found as a constituent of ambergris [18], oriental tobacco (*Demole, E.*, *Firmenich SA*, unpublished data), clary sage (*Renold, W.*; *Keller, U.*; *Ohloff, G.*, *Firmenich SA*, unpublished data) and ciste labdanum (*Renold, W.*; *Wuffli, F.*; *Ohloff, G.*, *Firmenich SA*, unpublished data). The absolute configuration of the natural (-)-form is determined by the configuration of the starting material (-)-sclareol **5**.

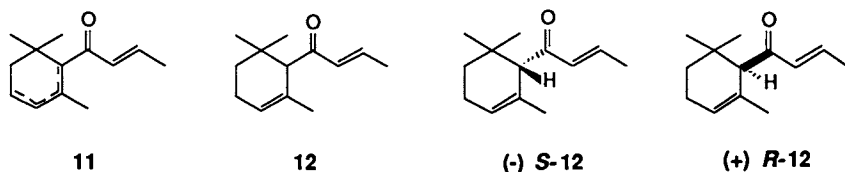
For the synthesis of the (+) enantiomer, eperuric acid **6** extracted from Wallaba wood (*Eperura falcata*) was converted to **7** following the method of Dey and Wolf [19]. The ketone **7** was then transformed into (+) Ambrox *ent*-**4**, following scheme 1 [20]. Optical purity, determined by 360 Mhz HNMR using Eu (hfbc)₃ as chiral shift agent, is more than 98%, confirmed also by capillary gas chromatography using Ni(hfbc)₂ in OV 101 as chiral stationary phase.

Scheme 1



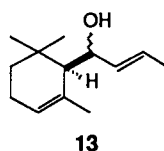
Reagents: a) O₂, t-BuOK, dry glyme (distilled over LiAlH₄);
 b) LiAlH₄, Et₂O/reflux/1 h;
 c) CH₃NO₂, TsOH/reflux/100°/1 h.

Organoleptic comparison of the two forms shows that the (+) enantiomer has a dominant woody note and lacks the warm animal note of the (-)-form. Thresholds in water [16] were measured to 0.3 ppb for (-) **4** and 2.6 ppb for the (+). The racemic mixture was determined to be 0.6 ppb, corresponding well to the calculated threshold of 0.54 ppb.

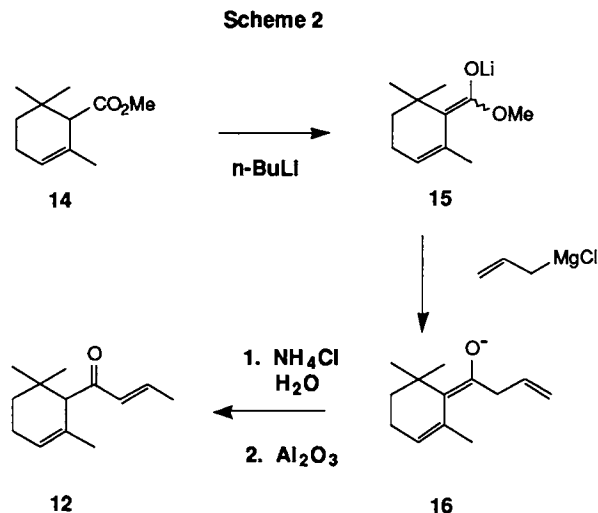


The rose ketones **11**, first discovered in 1970 [21] in Bulgarian rose oil, and named damascones, show unique organoleptic properties. Because of this they have elicited great interest, also as target molecules for new synthetic methods. α-Damascone **12** possesses a quite unique fruity odor, and its utilization allows the creation of perfumistic notes otherwise difficult to achieve.

Treatment of (+)-epoxy-α-dihydroionone with hydrazine hydrate gives as one of the reaction products alcohol **13**, which was transformed by oxidation with MnO₂ to (+)-(*R*)-α-damascone **12** in 65% e. e., thus establishing its absolute configuration [22].



A new access to α -damascone by selective kinetic protonation of α -ketone enolate, formed by reaction of an ester enolate with nucleophiles, has recently been described by Fehr and Galindo [23] (scheme 2).



The same authors found that the prochiral enolate **16** can, under certain conditions, be protonated enantioselectively, using ephedrine derivatives as proton sources [23]. These compounds are available in their optically pure forms, thus both enantiomers of α -damascone can be prepared in about 70% optical yield starting with the same ketone enolate and using the appropriate optical form of the proton source. Enantiomerically pure α -damascones (-)-(*S*)-**12**, (+)-(*R*)-**12** have been obtained by repeated recrystallization.

The organoleptic properties of the two compounds are distinct. Striking is the difference in perception thresholds, which were found to be 1.5 ppb for the (-)-(*S*)-, and 100 ppb for the (+)-(*R*)-form. Qualitatively, the (-)-(*S*) is described as more floral, reminiscent of rose petals, also having a winy character without the "cork" and the green apple note that are the characteristics of the (+)-(*R*)-form as well as of the racemic mixture.

These examples that add to the existing list show to what extent modification of the chemical structure of a molecule can alter the perceived odor. The fact that two enantiomeric forms of odorants show distinct differences in their organoleptic properties supports the hypothesis that the initial event, the interaction of the stimulus with the receptor is enantioselective, leading to diastereoisomeric stimulus-receptor complexes; and these events are transduced to give rise to different odor impressions, the mechanism of which remains to be discovered.

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Chapter 13

Role of Free Amino Acids and Peptides in Food Taste

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Free amino acids and/or some peptides have some sweetness, bitterness, sourness, saltiness and umami, and are very important as taste substances in foods. In this paper, we discuss 1) some tastes of free amino acids and some peptides, 2) the role of free amino acids in the characteristic tastes of vegetables and marine foods, 3) the role of the bitter peptides in cheese and the traditional Japanese foods "miso" and "natto", and 4) the contribution of free amino acids and peptides to the improvement of the meat taste during storage of meats (beef, pork and chicken).

Free amino acids and peptides are very important as taste substances. The importance of amino acids to food taste was first recognized by Ikeda in 1908 (1). He discovered that monosodium glutamate (MSG) was the essential taste component of traditional Japanese seasoners, such as sea tangle. MSG is a typical umami substance. Almost all free amino acids, including MSG, have some sweetness, bitterness, sourness and umami (2,3) and therefore contribute to the characteristic taste of foods. The characteristic taste of many marine foods is elicited by free amino acids. The taste of traditional Japanese foods such as sake, miso and soy sauce is thought to be caused by amino acids released from proteins during fermentation. Many studies on the taste of amino acids in foods and their production by extraction, fermentation, or chemical syntheses have resulted in mass production of various amino acids. Today, amino acids used in food processing not only enhance the nutritive value of many processed foods such as cereals, but also enhance the natural characteristic tastes of many foods.

Studies on the taste of peptides have been done only recently. The bitter taste produced during the storage of cheese and in the fermentation of the traditional Japanese food "miso" and "soy sauce" has been shown to be caused by the peptides in the hydrolysate of proteins. Since then, a number of studies on bitter peptides have

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been conducted. Also, research has been conducted on sweet and umami peptides recently.

This paper deals with the tastes of free amino acids and peptides, and their roles in the taste of foods.

For the convenience of our readers, the amino acid and peptide symbols used are:

Alanine	Ala	Leucine	Leu
Arginine	Arg	Lysine	Lys
Asparagine	Asn	Methionine	Met
Aspartic acid	Asp	Phenylalanine	Phe
Asn + Asp	Asx	Proline	Pro
Cysteine	Cys	Serine	Ser
Glutamine	Gln	Threonine	Thr
Glutamic acid	Glu	Tryptophan	Trp
Gln + Glu	Glx	Tyrosine	Tyr
Glycine	Gly	Valine	Val
Histidine	His	Anserine	Ans
Isoleucine	Ile	Carnosine	Car

TASTE OF FREE AMINO ACIDS

Amino acids are not only the building blocks of proteins but also occur in the free form. Amino acids commonly found in proteins have the L-configuration. Of these amino acids (Table 1), Asn was first discovered in asparagus in 1806, and Thr, the most recently discovered, was isolated from the hydrolysates of fibrin in 1935. Most of them were isolated from hydrolysates of various proteins. Glu, first obtained from wheat gluten hydrolysate in 1886, was found to be the most important taste component in sea tangle by Ikeda in 1908. Later, industrial production of MSG was undertaken to utilize it as a seasoner.

Almost all amino acids elicit taste. Most hydrophobic L-amino acids have a bitter taste. However, hydrophobic D-amino acids, which are formed simultaneously by the synthesis of L-amino acids, bring out a strong sweet taste. D-Trp, Phe, His, Tyr and Leu are 35, 7, 7, 6 and 4 times as sweet as sucrose, respectively (2). Gly and L-Ala elicit a strong sweet taste. It is thought that the strong sweet taste elicited by these amino acids is due to the ability of these molecules to bind to the sweet substance receptors.

L-Glu and Asp are sour stimuli in dissociated state, but their sodium salts dissociate on solution and elicit the umami taste. Free L-glutamate is contained in natural foods, as shown in Table 2 and contributes to the savory taste of foods as its sodium salt. Ibotenic and tricholomic acids (IA and TA) discovered in mushrooms are the derivatives of oxyglutamic acid and are also umami substances (4,5). The umami taste intensity of IA or TA is 4 to 25 times that of MSG. As these compounds are not amino acids commonly found in an animal system, they have not been used as seasoners. The umami taste of a MSG-, IA- or TA-5'-ribonucleotide mixture is much more intense than that of only MSG, IA or TA. Among 5'-ribonucleotides, 5'-inosinate and guanylate have synergistic effects in a mixture with MSG, IA or TA. This phenomenon is called the synergistic effect of

Table 1. Taste, Threshold Value and Discovery of Amino Acids

Amino acid	Taste	Threshold value(mg/dl)	Where found
His	Bitter	20	Casein and sturin*(1896)
Met	Bitter	30	Casein*(1922)
Val	Bitter	40	Albumin*(1879)
Arg	Bitter	50	Casein*(1895)
Ile	Bitter	90	Syrup(1904)
Phe	Bitter	90	Bean sprouts(1881)
Trp	Bitter	90	Casein*(1901)
Leu	Bitter	190	unknown(1819)
Tyr	Bitter	ND	Casein*(1846)
Ala	Sweet	60	Fibroin*(1875)
Gly	Sweet	130	Gelatin*(1820)
Ser	Sweet	150	Sericin*(1865)
Thr	Sweet	260	Fibrin*(1935)
Lys	Sweet and bitter	50	Casein*(1889)
Pro	Sweet and bitter	300	Casein*(1901)
Asp	Sour	3	Asparagine*(1827)
Glu	Sour	5	Gluten*(1886)
Asn	Sour	100	Asparagus(1806)
Gln	Flat		Beet(1883)
Cys		ND	Cystine(1884)

Glu Na	Umami	30	Sea tangle(1908)
Asp Na	Umami	100	unknown

ND, not determined; *, hydrolysate.

taste (6). When Gly was added to a MSG-5'-ribonucleotide mixture, the umami taste intensity of the mixture was greater than that of the mixture before addition (7). Ala, Cys, His, Met, Pro and Val, besides Gly, were also recognized as having the synergistic effect of taste in the mixture of MSG, 5'-ribonucleotide and free amino acids (8).

Though amino acids can elicit any one of the primary tastes, the threshold value of taste of each amino acid is high. As the levels of some free amino acids in natural foods are lower than their threshold values, it may be thought that they may not contribute directly to food taste. However, they may have an important role in making the food savory because of the synergistic effect.

TASTE OF PEPTIDES

Sweet Taste

The sweet peptide, aspartame (L-Asp-L-Phe-OMe) which has a sweet taste 180 times that of sucrose, was discovered by Mazur *et al.* (9). Aspartame is stable at pH 4 and unstable at pH 1 or 7-8. It is also unstable at high temperatures. Under these unstable conditions, the

Table 2. Free L-glutamate in Natural Foods

Food	L-glutamate (mg/100 g)
Kelp	2240
Parmesan cheese	1200
Green tea	668
Seaweed	640
Fresh sardine	280
Fresh tomato juice	260
Champignon	180
Tomato	140
Oyster	137
Potato	102
Chinese cabbage	100
Fresh shiitake mushroom	67
Soybean	66
Sweet potato	60
Dried sardine	50
Prawn	43
Clam	41
Chicken bones	40
Cabbage	37
Carrot	33
Bonito flakes	26
Pork fillet	23

ester linkage of aspartame may hydrolyze to produce aspartyl-phenylalanine (AP) or cyclize to produce the corresponding diketopiperazine (DKP). As none of these conversion products fits the sweet taste receptor, none of them is sweet.

Thaumatococcus (10) and monellin (11) are sweet and are proteins isolated from a plant native to Western Africa. Thaumatococcus and monellin are respectively 1600 and 3000 times sweeter than sucrose. As both proteins are basic, they are assumed to bind easily to the negatively charged taste cell.

There have been no reports regarding the detection of sweet peptides in naturally occurring foodstuffs other than thaumatococcus and monellin.

Bitter Taste

Almost all peptides of hydrophobic L-amino acids elicit a bitter taste, which indicates that the bitterness of peptides is caused by the hydrophobic property of the amino acid side chain. Ney (12) has reported that whether a peptide has a bitter taste or not depends on its hydrophobic value Q. The value Q is obtained by adding the Δf -values (Table 3) of each constituent amino acid residue of a peptide and dividing the sum by the number of amino acid residues (n).

$$Q = \frac{\sum \Delta f}{n}$$

If the value Q of a peptide is greater than 1400, the peptide

Table 3. Δf -value of the side chain of amino acid

amino acid	Δf (cal/mol)
Gly	0
Ala	730
Val	1690
Leu	2420
Ile	2970
Phe	2650
Pro	2600
Met	1300
Tyr	2870
Thr	440
Ser	40
Asp	540
Glu	550
Arg	730
Lys	1500
Trp	3000

will elicit bitter taste. This rule is applicable to almost all peptides.

Hydrolysis of proteins without taste by proteases often produces bitter peptides. Hydrophobic amino acid residues located in the interior of protein molecules in aqueous solution are exposed by fragmentation of the protein molecules treated with proteases, and the peptides containing a number of hydrophobic amino acid residues occur in the solution (13). Many bitter peptides as shown in Table 4 have been isolated from protein digests with proteinases (14-22).

The comparison of the amino acid sequence of the above-mentioned bitter peptides shows a large proportion of hydrophobic amino acids in each peptide. And the amino acid sequence of peptides also plays an important role in the intensity of the bitter taste. For example, the bitterness of Phe-Pro is more intense than that of Pro-Phe, and the bitterness of Gly-Phe-Pro is more intense than that of Phe-Pro-Gly (23). C-terminal groups of all bitter peptides in pepsin hydrolysates of the above-mentioned soy protein were characterized by the location of the Leu residue (14-17). The research on the relationship between the structure and bitter taste intensity of Arg-Gly-Pro-Pro-Phe-Ile-Val (BP-Ia) showed that Pro and Arg located on center and the N-terminal site, respectively, played an important role in the increment of bitter taste intensity besides the hydrophobic amino acids located on C-terminal site (24-26). This may indicate that the peptide molecular structure formed by the arrangement of Arg, Pro and hydrophobic amino acid residues contributes to the bitter taste intensity of the peptide.

Sour Taste

As shown in Table 5, dipeptides containing Glu and/or Asp, Gly-Asp-Ser-Gly, Pro-Gly-Gly-Glu and Val-Val-Glu in water elicit

Table 4. Bitter Peptides Isolated from Proteinase Hydrolysates of Proteins

Protein (proteinase*)	Peptides isolated from hydrolysate
Soy protein (pepsin)	Gly-Leu, Leu-Phe, Leu-Lys, Arg-Leu, Arg-Leu-Leu, Ser-Lys-Gly-Leu, PyroGlu-Gly-Ser-Ala-Ile-Phe-Val-Leu, Tyr-Phe-Leu, Phe-Leu
Zein (pepsin)	Ala-Ile-Ala, Ala-Ala-Leu, Gly-Ala-Leu, Leu-Gln-Leu-Leu-Glu-Leu, Leu-Val-Leu, Leu-Pro-Phe-Asn-Gln-Leu, Leu-Pro-Phe-Ser-Gln-Leu
Casein (papain)	Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln
Casein (trypsin)	Gly-Pro-Phe-Pro-Val-Ile, Phe-Phe-Val-Ala-Pro-Pro-Glu-Val-Phe-Gly-Lys, Phe-Ala-Leu-Pro-Glu-Tyr-Leu-Lys
Casein (bacterial proteinase)	Arg-Gly-Pro-Pro-Phe-Ile-Val, Val-Tyr-Pro-Phe-Pro-Pro-Gly-Ile-Asn-His, cyclo(Leu-Trp-Leu-Trp)

*, used to obtain hydrolysate of protein.

sour taste (3). This acidic sensation is assumed to be produced by the binding of the hydronium ion, produced by the dissociation of acidic amino acid, to the taste cell membrane.

Table 5. Sour Peptides

Gly-Asp, Gly-Glu; Ala-Asp, Ala-Glu; Ser-Asp, Ser-Glu;
Val-Asp, Val-Glu; Asp-Ala, Asp-Asp; Glu-Ala, Glu-Asp, Glu-Glu;
Glu-Phe^a, Glu-Tyr^a, γ -Glu-Gly^b, γ -Glu-Ala^b, γ -Glu-Asp^b, γ -Glu-Glu^b;
Phe-Asp, Phe-Glu, Trp-Asp, Trp-Glu;
Gly-Asp-Ser-Gly, Pro-Gly-Gly-Glu, Val-Val-Glu

All amino acids have the L-configuration. a, Accompanied by bitterness and astringency; b, Accompanied by astringency.

Salty Taste

It has been reported that there are salty stimuli in peptides. Tada *et al.* (27) inadvertently discovered the synthesized salty dipeptides, L-Orn- β -Ala·HCl, L-Orn-Tau·HCl, Lys-Tau·HCl and L-Orn-Gly·HCl having the same intensity taste as NaCl. The salty taste of L-Orn-Tau·HCl and Lys-Tau·HCl was more intense than that of L-Orn- β -Ala·HCl and L-Orn-Gly·HCl. The degree of dissociation of the carboxyl or sulfonyl group in peptides was assumed to contribute to the intensity of the salty taste. These dipeptides may be useful as new seasonings for diabetics and hypertensives because they contain no Na ions.

Recently, Huynh-ba and Philipposian (28) have reported that the L-Orn-Tau·HCl, L-Orn-β-Ala·HCl and L-Orn-Gly·HCl they synthesized elicited no salty taste. The salty taste of L-Orn-Tau·HCl synthesized by Tada *et al.* seemed to result from the NaCl present as an artifact in the method of preparation. However, the preparation of L-Orn-β-Ala·HCl (OBA·HCl) and L-Orn-Gly·HCl did not contain NaCl at all. The reason for this discrepancy is not clear yet. We heard from Okai group that the purified OBA without HCl did not elicit salty taste in water. When the molar ratio of HCl to OBA in OBA·HCl became 0.97 in the solution, this solution elicited a slightly salty taste. This salty taste elicited strongly with increasing HCl, till the molar ratio of HCl to OBA became 1.3 (Okai, H., Hiroshima University, personal communication, 1988.). As the molar ratio of HCl in OBA·HCl synthesized by Huynh-ba and Philipposian was 1.0, its salty taste might be very weak.

Umami Taste

Several dipeptides having L-Glu at N-terminus elicit the umami taste, though its umami taste intensity is much less than that of MSG. Arai *et al.* (29) synthesized L-Glu-X (X= amino acid) and examined their taste in aqueous solution containing NaCl at pH 6. Glu-Asp, Glu-Thr, Glu-Ser and Glu-Glu were found to produce the umami taste. Ohyama *et al.* (30) showed that Asp-Leu and Glu-Leu were umami substances. In section "Sour Taste", the peptides containing Asp or/and Glu were shown to elicit a sour taste in water. However, several of their peptides besides Glu-Asp and Glu-Glu may also be umami stimuli in aqueous solutions containing NaCl at pH 6.

When fish proteins were thoroughly hydrolyzed by pronase, the hydrolysate elicited the complex taste containing bitterness. Peptides having not only the bitter but also the umami taste were produced in this hydrolysate. The fraction of compounds with molecular weight under 500 was obtained from this hydrolysate by ultrafiltration. This fraction was divided into four fractions, aromatic, acidic, neutral and basic fractions. The acidic fraction had a very intense umami taste. Though Glu was removed from the acidic fraction by the treatment with ion-exchange chromatography, the treated acidic fraction also elicited an umami taste. Therefore, the umami taste of the acidic fraction can be ascribed in part to peptides, although MSG was mainly responsible for the umami taste. Umami peptides from this fraction (Table 6) were characterized by containing Glu residue and a number of hydrophilic amino acids except for Glu (31,32).

Table 6. Umami Peptides Isolated from Fish Protein Hydrolysates by Pronase*

Dipeptides:	Glu-Glu, Glu-Asp, Thr-Glu, Glu-Ser
Tripeptides:	Glu-Gly-Ser, Ser-Glu-Glu, Glu-Gln-Glu, Glu-Asp-Glu, Asp-Glu-Ser

*, Threshold values of these peptides are 150-300 mg/dl.

An octapeptide, Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala, which has a delicious taste, was isolated from beef treated with papain by the use of gel filtration and ion-exchange chromatography, and filter paper electrophoresis (33). A sensory evaluation showed that this octapeptide produced synthetically also elicited a delicious taste (34). The elimination of two amino acid residues at N-terminus, Lys-Gly, led to the disappearance of the savory taste and changed it into a sour taste. This indicated that the residues, Lys-Gly are important to the savory taste.

Other Tastes

Kirimura *et al.* (3) have reported that the dipeptides formed by the binding of γ -COOH group in Glu to NH_2 group in another amino acid, such as γ -L-Glu-Gly, γ -L-Glu-L-Ala, γ -L-Glu-L-Asp and γ -L-Glu-L-Glu, have not only sour taste but also astringent taste.

In general, because peptides were amphoteric electrolytes, they have a buffer action on taste. β -Ala-His (Car) and β -Ala-1-methyl-His (Ans) widely distributed in animal tissues were found to have a large buffer action in the pH range above 6.0 (35). Dipeptides, Gly-Leu, Pro-Glu and Val-Glu, were also found to have a buffer action (3). When these peptides were added to a synthetic "sake", a traditional Japanese alcoholic drink, composed of alcohol, glucose, succinic acid, lactic acid, phosphoric acid, NaCl, MSG, Gly and Ala, the buffer actions of sake containing Gly-Leu and Pro-Glu were larger in the pH range above pH 8 than that of sake without Gly-Leu and Pro-Glu. The buffer action of sake containing Val-Glu was larger in the pH range 7-9 than that of sake not containing Val-Glu. The buffer action seems to play an important role in the improvement of food taste by enhancing the taste of food and keeping the elicitation of its taste (36).

A glutamic acid-rich oligopeptides fraction was found to be effective in masking bitter taste (37). The addition of these peptides to the bitter medicinal drugs and drinks (summer orange and vegetable juices, and cocoa) seems to decrease or mask the bitterness of the products.

ROLE OF FREE AMINO ACIDS AND PEPTIDES IN FOOD TASTES

Vegetable Foods

Free amino acids play an important role in the taste of vegetables. There are large amounts of Glu, Asp, Ser, Val, Ala, Pro and Gln in vegetables as shown in Table 7 (38). The detailed research on the taste of green tea, onion and potato reveals the presence of umami substances. The most important umami substances of green tea are Glu and L-theanine, which is an ethylamide derivative of Glu (39-41). It has been shown that the most important umami substance of onion (42) is Glu. Buri *et al.* (43) examined the role of free amino acids in the flavor of boiled potatoes. Analytical data has shown that the taste of boiled potato soup stock was similar to that of synthetic potato soup composed of free amino acids and nucleotides. This indicated that free amino acids are very important in potato taste. Although they contain large amounts of Glu and Asp, there are

Table 7. Contents of Free Amino Acids in Vegetables
(α -amino N mg/100 g)

Amino acid	Tomato	Egg plant	Cucumber	Carrot	Pumpkin	Maize
Glu	3.99	0.84	0.65	3.02	3.03	0.33
Ser	10.07	0.59	2.85	-	2.28	0.55
Gly	4.83	0.48	0.54	-	0.82	0.44
Asn	6.61	2.66	3.33	2.34	9.77	1.32
Lys	1.89	-	-	-	1.16	-
Thr	0.11	+	0.35	0.33	0.46	0.22
Gln	6.58	3.02	-	1.44	5.09	4.18
Ala	0.92	0.97	0.95	1.33	2.24	1.32
Arg	6.81	2.05	0.53	4.11	1.95	1.32
Tyr	0.75	0.34	0.65	0.12	0.45	0.11
Val	9.17	2.35	1.10	1.23	4.01	3.52
Phe	2.37	-	0.65	-	2.05	0.33
Leu	2.14	0.95	0.95	0.45	0.74	0.77
Pro	-	0.54	0.50	0.55	1.71	1.76
Asp	-	0.99	0.80	2.88	-	0.66
Cys	-	0.50	-	-	-	+

+, trace; -, not detected.

Table 8. 5'-guanylate in Natural Foods

Food	5'-guanylate (mg/100 g)
Dried shiitake mushroom	156.5
Matsutake	64.6
Enokitake mushroom	21.8
Fresh shiitake mushroom	16-45
Truffle mushroom	5.8
Pork	2.5
Beef	2.2
Chicken	1.5

smaller amounts of nucleotides, IMP or GMP, in potatoes than in animal foods. In animal foods, IMP or GMP enhances umami and brothy taste elicited by MSG. This may account for the absence of the brothy taste in vegetables (44). However, mushrooms contain exceptionally high levels of GMP (Table 8). GMP in mushrooms enhances the umami taste of Glu by a synergistic effect and imparts a brothy taste.

Miso and natto are traditional Japanese foods made from soybeans by a fermentation process. These foods are produced by a mixed fermentation process using a characteristic microorganism and ripening for a given time. In these processes, the hydrolysis of proteins by microbial proteases results in the production of free

amino acids and peptides. Free amino acids and peptides produced in each food contribute to the characteristic taste of each food. The rate of liberation of Glu and Asp as umami substances during ripening of miso was very slow and amounts liberated from proteins were small (45). Examination of the change in peptides during storage of miso showed that the peptides of A.P.L. (the average number of amino acid residues in the peptide) 3-4 gradually decreased, but the peptides of A.P.L. 3-4 rapidly increased during the initial stages of storage. The peptides of A.P.L. 13-20 increased with storage time (Fig. 1) (46). As 40 % of the constituent amino acids residue in the peptides of A.P.L. 13-20 was Glu, these peptides seemed to play an important role in the umami taste of miso.

The free amino acid content in natto was very small and corresponded to only about 10 % of the total nitrogen compounds. Most of the other nitrogen compounds were peptides. These peptides have been shown to contribute to the bitterness of the characteristic taste of natto (47). One of these peptides was isolated and its amino acid sequence was investigated. The amino acid composition of this peptide was Asp 1, Thr 1, Glu 1, Ala 1, Pro 2, Val 3, Ile 3 and Leu 5. The amino acid at N-terminus of this peptide was Leu and the C-terminal structure was -Ala-Val-Ile-Leu.

A cyclic dipeptide, Pro-Leu anhydride, having bitterness was isolated from a traditional Japanese alcoholic drink "sake" (48). This peptide increased the longer sake was stored in sake production. So this peptide seems to contribute to the bitter taste of sake.

Animal Foods

Taste components of a number of sea food products have been examined for each sea food product has its individual characteristic taste. In studies on the free amino acids analysis (Table 9), it was shown that the major amino acid is His in red meat of fish, Gly and Pro in cuttlefish, Gly and Arg in prawns, and Tau and Arg in abalone (49). The major amino acids in sea urchin are Gly, Ala and Leu (50). However, the components contributing to their individual characteristic tastes were not elucidated because the relationship between the taste components and the taste was not thoroughly correlated and investigated in most of these studies.

Detailed research on the relationship between the taste components and the taste of sea urchin, shrimp and crab led to the identification of the characteristic taste components. The characteristic components of sea urchin are Gly, Ala, Val, Glu, Met, inosine 5'-monophosphate (IMP) and guanosine 5'-monophosphate (GMP) (51). The contribution of Gly and Ala to sweetness, Val to bitterness, and Glu, IMP and GMP to umami taste was found. Met was shown to be responsible for the characteristic taste of sea urchin. The characteristic taste of shrimp is sweet taste which is attributed to Gly, the largest component of all the free amino acids in shrimp (52). The 12 components - Gly, Ala, Arg, Glu, CMP, AMP, GMP, Na⁺, K⁺, Cl⁻, PO₄³⁻ and betain - were shown to contribute to the characteristic taste of boiled crab extract (53).

Free amino acids and peptides released by such proteolytic enzymes as chymosin and lactic acid bacterial proteases in cheeses contribute to the formation of cheese taste. Biede and Hammond (54) reported that free amino acids and small peptides played an important

Table 9. Contents of Free Amino Acids in Sea Foods (mg/100 g)

Amino acid	Plaice	Yellowfin tuna	Cuttlefish	Abalone	Scallop	Prawn	Snow crab
Tau	171	26	160	946	176	150	243
Asp	+	1	+	9	+	+	10
Thr	4	3	9	82	38	13	14
Ser	3	2	27	95	6	133	14
Gln + Asn	1	-	-	-	-	-	+
Glu	6	3	3	109	99	34	19
Pro	1	2	749	83	36	203	327
Gly	5	3	832	174	613	1222	623
Ala	13	7	181	98	82	43	187
Cys	-	-	3	-	3	+	-
Val	1	7	3	37	10	17	30
Met	1	3	7	13	12	12	19
Ile	1	3	6	18	3	9	29
Leu	1	7	12	24	0.3	13	30
Tyr	1	2	8	57	2	20	19
Phe	1	2	2	26	4	7	17
Trp	-	-	5	20	-	+	10
His	1	1220	16	23	10	16	8
Lys	17	35	15	76	7	52	25
Arg	3	0.6	246	299	935	902	579

+, trace; -, not detected.

role in producing the sweet and brothy tastes of Swiss cheese, and that medium sized (tri to hexa) peptides played an important role in bitterness. A number of studies on bitter peptides of cheese have been carried out. Several bitter peptides were isolated from different cheeses and their structures were determined. L-Leu-Trp-OH, a bitter peptide, was isolated from Swiss cheese (55), and Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser from Butterkäse (56). From Cheddar cheese were isolated Pro-Phe-Pro-Gly-Ile-Pro, Pro-Phe-Pro-Gly-Pro-Ile-Asn-Ser, and Gln-Asp-Lys-Ile-His-Pro-Phe-Ala-Gln-Thr-Gln-Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro (57). Recently, the formation mechanisms of free amino acids and peptides contributing to cheese taste have been shown (58). As shown in Fig. 2., the peptide, α SI-CN(f1-23), obtained from Gouda-type cheese was produced by the action of chymosin with α SI-casein(-CN). This peptide was degraded by lactic acid bacterial protease and small peptides, including three major peptides, α SI-CN(f1-9), α SI-CN(f1-13) and α SI-CN(f1-14), were formed. These small peptides were further degraded into smaller peptides and free amino acids by aminopeptidase of lactic acid bacteria.

It is said that flavor of beef, pork and chicken is improved by storage at a low temperature for given periods. We examined the effect of the storage at low temperature on the taste of meats (59). After beef, pork and chicken were stored at 4°C for 8, 5 and 2 days, respectively, the changes in intensity and levels of brothy taste and

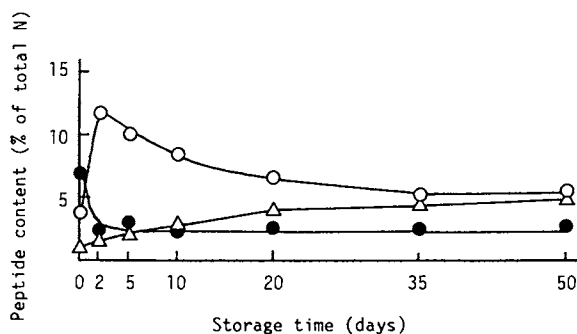


Fig. 1. Changes in various peptides during the storage of miso. A.P.L., the average number of amino acid residues in the peptides.

○—○, A.P.L. 3-4; ●—●, A.P.L. 4-6; △—△, A.P.L. 13-20.

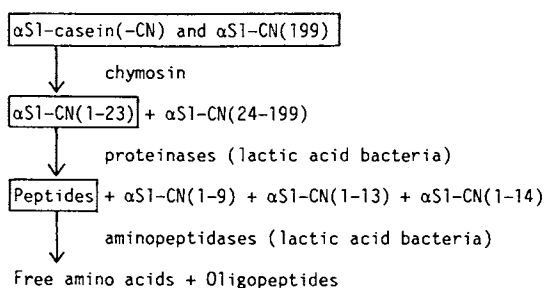


Fig. 2. Pathway of α S1-casein degradation during Gouda-type cheese ripening.

□, protein or peptides subjected to hydrolysis.

taste components were examined. Examination of the brothy taste intensity of meat before and after storage showed that the brothy taste intensity of pork and chicken was significantly stronger after storage than before. There was no significant difference in the brothy taste intensity before and after storage of beef (Table 10).

Table 10. Effect of Additional Storage on the Intensity of the Brothy Taste of Beef, Pork and Chicken

Meat	The no. of samples judged to have a more intense brothy taste :		n	Difference ^a
	Before additional storage	After additional storage		
Beef	12	4	16	NS
Pork	2	14	16	*
Chicken	8	23	31	*

a: NS, not significant; *, significant ($p < 0.05$).

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The changes of various taste components of meats during storage were also determined. In pork and chicken, especially, the differences in the levels of free amino acids and peptides before and after storage were very large (Fig. 3, 4), and these results corresponded to the higher brothy taste intensity in pork and chicken after storage of meat as compared to before storage. This correspondence suggests that the increase in free amino acids and peptides contributed to the improvement of meat taste after storage.

Sensory evaluation of the relative strengths of each taste (sweet, sour, umami, salty, bitter and brothy) among beef, pork and chicken soups prepared after storage showed that the intensity of umami and brothy tastes was weakest in beef soup (Fig. 5) (Rhue, M.R., University of Tokyo, unpublished data.). There was less Glu in beef than in pork and chicken. The addition of Glu into beef soup to bring up the Glu concentration equal to those in pork and chicken soups made the umami and brothy tastes in the beef soup similar to those in pork and chicken soups. From this observation, Glu seems to play a very important role in the umami and brothy tastes of meats. This experiment showed that other free amino acids also contribute somewhat to the meaty taste.

The role of peptides in the taste of meats after storage is not at all clear though peptides seem to contribute to the improvement of the taste of stored meat as mentioned above. The role of Car and Ans found in significant amounts in meats is also unknown. These problems must be studied for clarification.

Each food has a characteristic taste which is determined by the balance of the primary and/or secondary tastes. Free amino acids and peptides play an important role in the elicitation of each food taste.

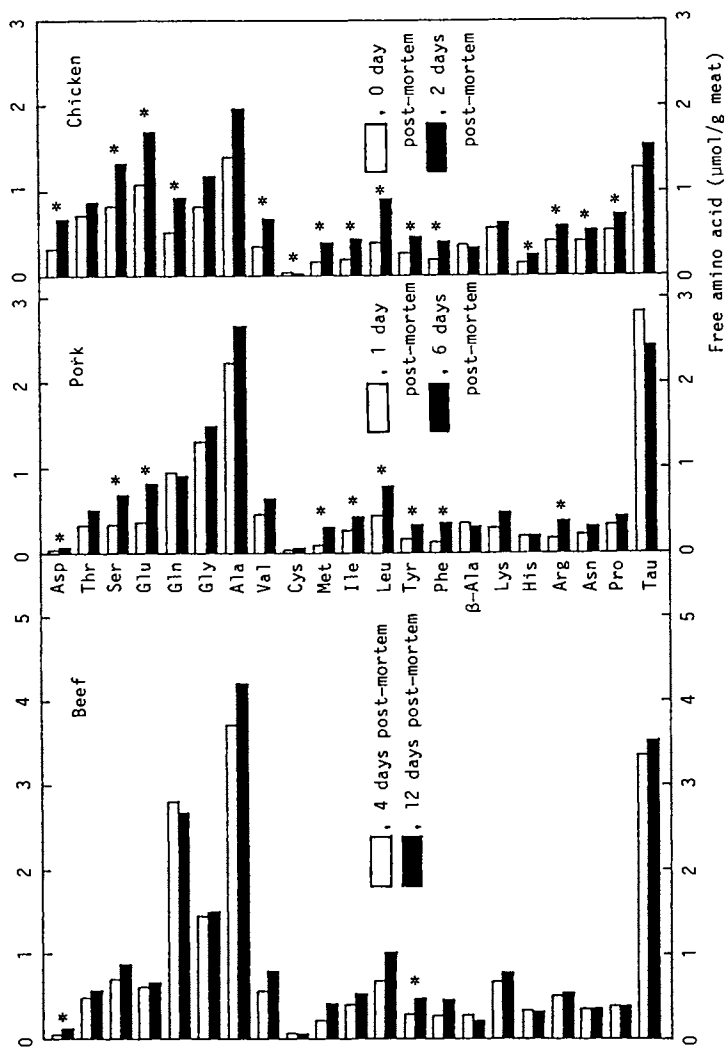


Fig. 3. Free amino acids contained in heated soup of beef, pork and chicken before and after additional storage. *, significantly different ($p < 0.05$). (Reprinted with permission from ref. 60. Copyright 1988 Marcel Dekker.)

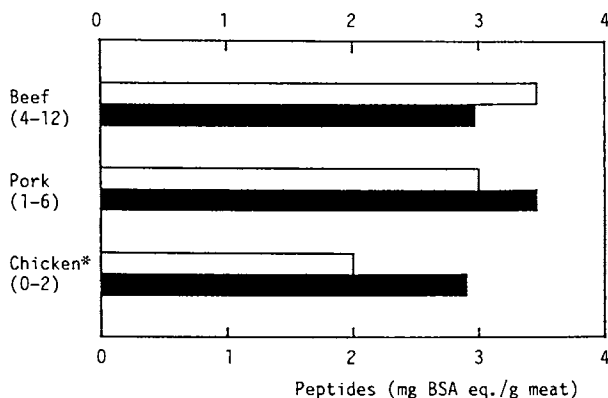


Fig. 4. Peptides contained in heated soup of meat before and after additional storage. The levels of peptides were obtained from the difference between the values of phenol reagent-positive materials before and after the addition of Cu^{2+} into the phenol reagent. , before additional storage; , after additional storage; *, significantly different ($p < 0.05$); numbers in parentheses, time post-mortem (days). (Reprinted with permission from ref. 60. Copyright 1988 Marcel Dekker.)

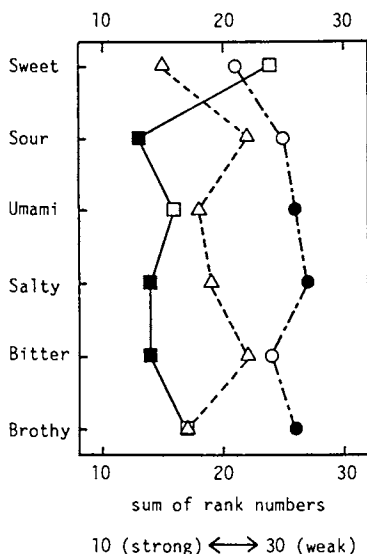


Fig. 5. Relative strength of each taste among beef, pork and chicken soups. The NaCl concentration of each soup was adjusted to 0.508%. \bigcirc — \bigcirc , beef; \triangle — \triangle , pork; \square — \square , chicken. Closed symbols, significantly different from others ($p < 0.05$). (Reprinted with permission from ref. 60. Copyright 1988 Marcel Dekker.)

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Chapter 14

New Dimensions in Flavor Research

Herbs and Spices

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Herbs and spices are not only common household food ingredients but also integral parts of various flavor and fragrance creations. Most of the spices used day-to-day are either dried or aged, but few people are aware of the fact that live spices have different aroma properties from those that are dead. The chemical differences in the aroma profiles of "living" vs dead leafy spices have now been characterized. The methodology and analytical results for some common spices are presented.

Today, in western society, we take herbs and spices for granted, but this was not true in the past where they were rare and prized commodities. Herbs and spices were so precious that even a slave could be bought for a handful of spice (1).

Generally speaking, the important spices came from the East, especially from India, Ceylon, and the eastern Spice Islands. Beginning with Marco Polo, various travelers like Vasco da Gama ventured eastward, found the lands of spice, and opened the door to the West for the spice trade. England eventually became the center for the European spice trade. It should be mentioned in this connection that, in the late 17th Century, the Americans also benefited from the spice trade. Boston-born Elihu Yale went to England where he worked in the British East India Company which held a monopoly on all trade with India and whose ships brought the first cargo of cinnamon. He eventually became Governor of Madras, India and acquired a fortune which he donated to a university in Connecticut which now bears his name and is known as Yale University (1).

From ancient times up until modern days herbs and spices have played a dynamic role in our daily lives. When we clean our teeth in the early morning with toothpaste we encounter mint oils. When we wash our bodies and clothes with soaps and detergents we find the essences of rosemary and lavender. At midday and in the evening on the dinner table the smells of spices elevate the appetite. More people than ever are discovering the secrets of great cuisine which rely heavily on herbs and spices. In addition

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to the use of herbs and spices in the culinary arts, the back-to-nature health movement has also called our attention to these materials. People are buying more and more herb and spice products, however, they are not aware of the fact that most of these products have been processed and the aromas are entirely different from those found in the living plant. The reason is that when the umbilical cord connecting the fruit, flower, leaf, or seed to the plant is severed, these products are then essentially dead and their aroma is perceptibly changed.

The chemical differences in the aroma profile between living and dead fruits, flowers, herbs, and spices have now been characterized, and the methodology and analytical results for several common examples will now be described. The first example will deal with typical results obtained for a living vs dead flower. The first flower chosen not only has the highest priority in the fragrance industry but also is heavily used for flavoring foods by Oriental people. This flower is none other than the rose which was called the "Queen of Flowers" by the Greek poetess, Sapho, in 600 B.C. The rose most likely originated in China and was introduced into Spain from China by invading Arabs in the 7th Century and into India in the 10th Century (2). The rose is prized chiefly for its blossoms, and, though it has been known since ancient times for the making of fragrance, it was Empress Nurjuhan, the wife of Indian Emperor Jahangir, in the 13th Century who first made attar of rose by spreading rose petals on her morning bath water.

Of the 200 varieties of rose the most coveted for the making of Otto of Rose for fragrance use is Rosa-damascena which comes from Bulgaria. It takes about 4000 pounds of rose to produce one pound of Rose Otto; hence the cost of \$2500 per pound. Although most roses grown for commercial oil production come from Bulgaria or the south of France, in the 1930's American horticulturists started to breed hybrid tea roses for both their form and fragrance. Many of them have unique aromas in their own right, although none is the equal of Rosa damascena. One of the best from the point of view of aroma, yellow tea rose (J.F.K.), was chosen for analysis.

Two side-by-side experiments were performed on the yellow tea rose; first on the picked blossoms and next on the blossoms still attached to the plant. The latter is called the "living flower" analysis. (Duplicate experiments were performed on other blossoms from the same plant and on blossoms from other plants of the same species and variety. In all cases, there were no significant differences observed in the analytical data.)

In the method of analysis routinely employed on picked flowers, the blossoms are placed in a flask equipped with a trap packed with Tenax GC. The flask is purged with air for 6-12 hours depending on the type of flower, and the volatiles are collected on the Tenax and then desorbed into the gas-liquid chromatograph for analysis by GC/MS. In the method of analysis used for the living flower, one single living blossom is placed into a suitable glass chamber which contains a Tenax trap on one sidearm. Air is drawn over the blossom and through the Tenax trap by a pump under the same conditions as employed for the picked flower. In this way, the aroma profiles of many different flowers were compared.

The comparative headspace analysis of living vs picked yellow tea rose (J.F.K.) is shown in Table I.

Table I. Major Differences Between Living and Picked Yellow Tea Rose (J.F.K.) Flower

Compound	Living Rose Flower % (AN)	Picked Rose Flower Air Purged % (AN)
cis-3-Hexenyl acetate	20.7	5.4
Hexyl acetate	8.4	4.3
Phenyl ethyl alcohol	5.7	3.3
Phenyl ethyl acetate	5.5	1.5
3,5-Dimethoxy toluene	10.0	18.6
alpha Elemene	-	4.1
Geranyl acetone	2.2	-
Dihydro beta ionol	-	2.6
alpha Caryophyllene	0.3	2.1
alpha Farnesene	5.8	3.0

These data reveal that the composition of the picked tea rose is very much changed from that of the living rose. As one can see, cis-3-hexenyl acetate which constitutes 21% of the living rose headspace volatiles is drastically reduced to 5% in the picked rose. At the same time, 3,5-dimethoxy toluene, one of the character-donating components of tea rose, is dramatically doubled in the picked flower, whereas important constituents like phenyl ethyl alcohol and its acetate are reduced in the picked flower.

In this way, many other common and uncommon flowers such as jasmine, narcissus, osmanthus, honeysuckle, hyacinth, lily-of-the-valley, lilac, and tuberose have been analyzed. In all cases, considerable differences have been observed in the aroma profiles of the living and picked flowers. In connection with the living flower analytical program, the concept was also extended to the flavor field, especially fruits. The fruits and flowers work has served as the basis of a recent report to the 10th International Congress of Essential Oils in Washington, D.C. in 1986.

The first subject to be tested in the fruits area was peach due to the importance of its flavor and aroma to both the flavor and fragrance industry. The peach actually originated in China though botanists thought that it came from Persia, hence its name, *Prunus persica* (Persian Plum-Tree). From China, its cultivation spread west to Persia in the 3rd Century B.C. eventually reaching Europe. From there, the Spanish introduced the peach to the New World where the American Indians developed a taste for the fruit, even naming one of their thirteen months for it. Thomas Jefferson, a peach lover, planted peach trees at his birthplace, Monticello, in Virginia, when he became President (3). This is a brief history of the peach, and now the analysis of the living peach will be described.

A peach still attached to the tree was selected for analysis on the basis of its possessing a full, rich, at-the-peak-of-ripeness aroma. Taking care not to bruise the fruit, the peach

was placed into a flask which was designed to handle larger objects. With the Tenax trap and pump in place, the respiration gases of the fruit were collected for 16 hours. A peach of equal ripeness was harvested from the same tree and immediately set up for collection of its volatiles. The major differences between the headspace volatiles of living and picked peach are shown in Table II.

Table II. Major Differences Between Living and Picked Peach

Compound	Living Peach	Picked Peach
	% (AN)	Air Purged % (AN)
Ethyl acetate	6.2	-
Dimethyl disulfide (a)	0.6	-
cis-3-Hexenyl acetate	9.7	-
Methyl octanoate (a)	34.2	7.1
Ethyl octanoate	7.4	11.0
6-Pentyl alpha pyrone	trace	10.6
gamma Decalactone	2.5	39.2

(a) Identified for the first time in peach

One can observe that the major volatiles of living peach are lower boiling with methyl octanoate, now identified for the first time in peach, predominating. The identification of dimethyl disulfide for the first time in peach is of interest. Very little peach lactone and pentyl pyrone are seen in the living peach, whereas they are major components of the picked fruit. Methyl octanoate is considerably decreased and the lower boiling constituents are essentially gone after picking.

It is reasonable to expect that what is true for living and picked fruits and flowers could also be true for herbs and spices, although it is possible to keep herbs and spices in an acceptable olfactory condition for longer periods of time than one can preserve picked fruits and flowers. The first subject for testing of this theory was mint because of its extensive use in the flavor industry. American spearmint, *Mentha spicata*, will be described first. It is interesting to note that the word "mint" was coined by the early Greeks after the mythical character MINTHE. The term "mint" refers to the dried leaf of the spearmint plant, which, available in flake or extract form, has an aromatic, sweet flavor with cool aftertaste (4).

Interestingly, American spearmint is not native to North America but was introduced from Europe during the 17th Century and has since been widely grown. Millions of pounds of this oil have been produced in this country due to its extensive and popular use as a flavoring ingredient, particularly in chewing gums and toothpastes.

The technique of headspace analysis of the living and picked American spearmint plant is the same as in the case of living and picked flowers and fruits. The picked spearmint was taken from the same plant used for the living plant analysis. In order to

simulate the commercial process for making spearmint oil, freshly picked stems and leaves were kept at room temperature for 24 hours with a weight loss of 50%. This semi-dried material was then analyzed for headspace volatiles and compared with that of living plants. Table III represents the comparative analysis of living vs picked spearmint plant and, for purposes of comparison, a typical commercial oil.

Table III. Major Differences in Spearmint Volatiles

Commercial	Living Plant	Picked Plant	Comm. Oil
	% (AN)	Air Purged % (AN)	
Hexanal	0.5	trace	-
Hexanol	-	2.3	0.1
Limonene	17.7	1.8	21.4
Dihydro carvone	0.7	2.6	0.1
Carvone	24.0	70.0	63.0
Menthone/isomenthone	-	-	1.2
Menthol isomers	-	-	1.7
1,3,5-Undecatriene (a) (mixture of 4 isomers)	0.5	-	-

(a) Identified for the first time in spearmint volatiles

Interestingly, neither the isomeric menthones nor the isomeric menthols were detected in the living or picked plant material, but they are both present in appreciable amounts in commercial oil. At the same time, a very powerful green odorous compound, 1,3,5-undecatriene (isomer mix), has now been identified for the first time in the living spearmint to the extent of 0.5%. One can also observe that carvone, the true character-donating component of spearmint oil, constitutes 70% of the total headspace volatiles of picked spearmint but only 24% of those of the living plant. The opposite is true in the case of limonene which is only a minor constituent in the picked plant but a major component of the living mint. These variations in constituents will drastically influence the odor of living spearmint.

After spearmint, naturally comes peppermint, Mentha piperita, as distinguished from the many other species of Mentha herb including Mentha spicata. This herb is native to Europe and has become naturalized in North America. Of the many hybrids of peppermint, only two varieties, black and white, are commonly grown. Of these, black peppermint, also known as English peppermint, is the variety most extensively grown in the United States because of its hardness and high oil yield. The United States is the world's largest peppermint oil producer, and the oil is mainly and extensively used for oral hygiene products, chewing gum and confectioneries. Pure peppermint oil has a very agreeable odor and a powerful, aromatic taste followed by a sensation of cold when air is drawn into the mouth (5). Table IV represents the comparative analysis of living vs picked peppermint plant and commercial oil. These experiments were performed on black peppermint.

Table IV. Comparative Analysis of Peppermint Volatiles

Compound		Living Plant	Picked Plant	Comm. Oil
		% (AN)	Air Purged % (AN)	
Hexanal	(a)	-	0.1	-
cis-3-Hexenal		-	0.5	-
trans-2-Hexenal		-	0.8	-
cis-3-Hexenol	(a)	-	0.3	-
trans-2-Hexenol	(a)	-	1.4	-
Hexanol	(a)	-	0.5	-
2,4-Hexadienal	(a)	-	0.1	-
1-Octen-3-ol	(a)	-	2.0	-
Eucalyptol		-	-	5.7
Menthone		0.2	12.7	18.1
isoMenthone		9.6	7.7	2.3
Menthofuran		49.7	26.3	5.2
neoMenthol		-	-	1.7
Menthol		trace	4.7	44.2
neoisoMenthol		-	-	1.9
isoMenthol		-	-	0.2
Pulegone		1.6	24.5	1.7
1,3,5-Undecatriene (a) (mixture of 4 isomers)		0.6	-	

(a) Identified for the first time in peppermint volatiles

As one could see, tremendous differences exist between the living and picked peppermint volatiles. For example, six-carbon alcohols and aldehydes are present only in the picked plant. Interestingly, these very green compounds have not been identified before in peppermint. At the same time, 1-octen-3-ol, which possesses an earthy, mushroom odor and which has not been previously found in peppermint, was identified only in the picked herb. In the class of compounds responsible for the cooling effect of peppermint oil, surprisingly, menthone is a major component in the picked herb but is only present in trace quantities in the living plant, whereas isomenthone, the more powerful of the two isomers, is present to approximately the same extent in both. Menthofuran, which has the reputation of being a less desirable component of mint oils, surprisingly constitutes 50% of the volatiles of the living plant decreasing to 26% in the picked plant material. In the opinion of the present authors, menthofuran imparts a characteristic fresh mintiness. On the other hand, menthol, the classical cooling compound, is present in negligible amounts in the living plant while its content varies from 5-45% in picked and commercial oils respectively. Pulegone, another characteristic component with a sweet, weedy, minty odor, is a major component only in the picked peppermint. Finally, as with spearmint, the powerful, diffusive herbaceous - green compound, 1,3,5-undecatriene (isomer mix), was detected only in the living peppermint and at a relatively high level (0.6%) considering its strength. This compound has never before been identified in peppermint. Now, one can easily see that a living

peppermint leaf has quite a different aroma from that of dried peppermint or commercial peppermint oil.

The next herb which was analyzed was rosemary, Rosmarinus officinalis, not because the herb finds extensive use as a seasoning, condiment, or meat flavor, which it does, but because it is widely employed in fragrancng colognes, toilet waters and household products such as soaps and detergents. Rosemary is rich in legend and tradition. It is said to have been used as early as 5000 B.C. One legend claims that rosemary will grow only in the gardens of the righteous (6). Even in Shakespeare's "Hamlet", Ophelia said, "There is rosemary; that's for remembrance." In England to this day rosemary is placed on the graves of heroes so that their memories will be eternal. Its name is derived from "ros maris" which means "sea-dew" (7), and it indeed grows near the sea in Spain, Dalmatia, Morocco, Tunisia, and Turkey.

Table V shows the comparative analysis of the headspace of living and picked rosemary and a typical commercial oil.

Table V. Comparative Analysis of the Volatiles of Rosemary

Compound		Living Plant	Picked Plant	Comm. Oil
		% (AN)	Air Purged % (AN)	
trans-2-Hexenal	(a)	-	0.2	-
cis-3-Hexenol	(a)	-	0.7	-
Hexanol	(a)	-	0.3	-
alpha Pinene		1.1	0.7	13.3
Myrcene		9.5	11.1	1.7
beta Pinene		0.2	0.1	7.6
para Cymene		19.8	13.7	1.7
Limonene		14.1	14.3	1.0
Eucalyptol		2.0	0.7	44.5
Linalool		7.1	7.6	0.8
Camphor		0.2	-	10.1
Estragole	(a)	3.0	0.3	trace
cis-Carveol	(a)	0.2	0.6	-
Citronellol	(a)	0.6	1.1	-
alpha Campholenic alcohol and acetate	(a)	0.3	0.8	-

(a) Identified for the first time in rosemary volatiles

Again, one could easily see the qualitative and quantitative differences between the living and picked rosemary. It is interesting to observe that the very fatty-green components, trans-2-hexenal, cis-3-hexenol, and hexanol, are present only in the picked plant and were not detected in the living herb. At the same time, hydrocarbon constituents, alpha pinene, myrcene, beta pinene, para cymene, and limonene, do not vary much from living to picked. However, the most interesting observation from our experiment is that eucalyptol and camphor, which traditionally are the major constituents of commercial rosemary oil as shown here, (45 and 10% respectively), are present in both living and picked rosemary only in very small quantities, 0.7-2% for eucalyptol and

0.2% for camphor which was not found in the picked plant at all. The present authors are, therefore, of the opinion that eucalyptol and camphor do not play major roles in producing the fresh rosemary odor whereas estragole, a newly reported rosemary constituent possessing a powerful sweet, herbaceous aroma, probably is in large part responsible for the fresh herbaceous rosemary character. It is also of interest to note the identification for the first time in rosemary of *cis*-carveol, citronellol, and alpha campholenic alcohol and its acetate and to observe that the quantities of each increase on picking.

The next herb chosen for analysis was thyme, *Thymus vulgaris*, which is also native to southern Europe and the Mediterranean and is cultivated in the southern United States as well. Thyme is used extensively in flavors for food products such as sauces, dressings, pickles, and canned meats as well as in pharmaceutical preparations. The excellent germicidal properties of the phenols of the oil are exploited in oral hygiene products such as gargles and mouthwashes and in numerous disinfectants. Cough syrups and lozenges are often activated with thyme oil. In perfumery, it finds use in soaps and detergents for its freshness with hints of medicinal notes. Table VI shows the comparative analysis of the headspace volatiles of thyme.

Table VI. Comparative Analysis of the Volatiles of *Thymus vulgaris*

Compound	Picked Plant		Comm. Oil
	Living Plant % (AN)	Air Purged % (AN)	
<i>trans</i> -2-Hexenal (a)	-	2.8	-
2,4-Hexadienal (a)	-	0.1	-
<i>cis</i> -3-Hexenyl acetate (a)	11.2	0.1	-
1-Octen-3-ol	8.2	8.0	-
<i>para</i> Cymene	30.0	50.0	30.0
Limonene	1.3	1.1	1.7
Thymol methyl ether	-	1.3	-
Carvacrol methyl ether	-	1.5	0.1
Thymol	15.2	9.0	39.7
Carvacrol	1.5	0.9	1.0

(a) Identified for the first time in thyme volatiles

As has been observed in the case of peppermint and rosemary, fatty-green six-carbon components like *trans*-2-hexenal and 2,4-hexadienal are again found only in the picked thyme and are not detected at all in the living plant. It may be concluded that these components are actually formed by enzymatic oxidation during the overnight drying process. The fresh herbaceous quality of the living plant is probably not associated with these components, but is, at least in part, due to the true green aroma of compounds like *cis*-3-hexenyl acetate which occurs to the extent of 11% in the living plant but is only a trace component of the picked herb. At the same time, the characteristic aroma components of thyme oil, thymol and carvacrol, are both present to a greater extent in

the living herb. Interestingly, the corresponding methyl ethers are detected only after picking. However, para-cymene increases from 30-50% on picking.

Evergreen Cassia, Cinnamomum cassia also known as Chinese Cinnamon, is native to China, Burma, and many sub-tropical countries. It has a long, shiny leaf, a small pale green flower, and a loose peeling bark. The trees are grown in plantations and are coppiced for the new long shoots which provide the scented bark. The stripped bark curls into quills as it dries and is exported in bundles. The dried unripe fruits are sold as Chinese cassia buds, and the dried leaves and stems are used to distill cassia oil. Totally dried leaves are used as a flavoring "Tej Pat" in the day-to-day Indian cookery. Cassia has been used as a spice in Europe since the Middle Ages and it has also found use in the treatment of indigestion and to increase the flow of mother's milk (8). We chose to compare the volatiles of fresh leaves and aged leaves dried to 50% weight along with commercial oil. The results for cassia leaves freshly harvested in Hawaii are shown in Table VII.

Table VII. Comparative Analysis of Volatiles of Cassia Leaf

Compound	Fresh Leaf % (AN)	Aged Leaf Air Purged % (AN)	Comm. Oil
trans-2-Hexenal	(a) 4.0	0.8	-
Phenyl ethyl alcohol	2.1	0.1	0.4
trans-Cinnamaldehyde	50.0	70.0	70.0
Cinnamyl alcohol	20.6	0.3	0.3
Coumarin	7.9	4.4	1.7
2-Methoxy cinnamaldehyde	1.0	1.4	11.5
4-Methoxy cinnamaldehyde (a)	4.1	12.3	-

(a) Identified for the first time in cassia volatiles

Table VIII shows the dramatic differences between fresh and dried leaves. In this case, as opposed to peppermint, rosemary and thyme, trans-2-hexenal is more in the fresh than in the dried and aged. The same is true for phenyl ethyl alcohol. Interestingly, trans-cinnamic aldehyde constitutes 50% of the total living headspace volatiles, but it is still less than in the aged leaf and commercial oil. However, cinnamyl alcohol represents 20% of the fresh volatiles but is only a trace component of the aged leaf and oil. 4-Methoxy cinnamic aldehyde, identified as a cassia constituent for the first time, also increases 3-fold on drying but has disappeared completely in the commercial oil. 2-Methoxy cinnamic aldehyde, sometimes called the character impact component of cassia oil, is present in the headspace of the leaves to only a minor extent but it is the second most abundant component of the oil.

An herbal plant, a part of which is also considered as a spice, is the last subject to be described. This plant is coriander, Coriandrum sativum. Even though the name, "coriander" originates from the Greek "koriannon" meaning "bug", a reference

to the smell of the leaves and unripe seed, actually it has been cultivated for thousands of years in India, China and Egypt (8). Even today the seeds and leaves are used daily in Oriental countries for flavoring cooking. But, at the same time, oil derived from dried seeds is an important ingredient in modern perfumery, particularly in fine fragrances such as "Drakkar Noir".

Since, in the opinion of the present authors and contrary to the observations of the ancient Greeks, green coriander leaves and seeds have a very aesthetic aroma, the odor profiles of both living and dead leaves and seeds were compared. The data for coriander leaf volatiles are shown in Table VIII.

Table VIII. Comparative Analysis of Volatiles of Coriander Leaves

Compound		Living Leaf % (AN)	Picked Leaf Air Purged % (AN)	Comm. Herb Oil
Hexanal	(a)	0.4	2.0	trace
trans-2-Hexenal	(a)	0.3	3.2	-
Hexanol	(a)	-	1.1	trace
Nonane	(a)	15.2	4.7	3.6
Decanal		11.4	4.7	14.8
trans-2-Decenal	(a)	35.5	39.2	26.8
trans-2-Decenol	(a)	2.6	-	2.4
Decanol	(a)	2.5	-	1.3
Undecanal		1.5	-	0.7
2,4-Decadienal	(a)	0.1	-	-
Dodecanal		1.2	-	0.5
trans-2-Dodecenal	(a)	9.7	4.3	2.7
trans-2-Dodecenol	(a)	0.4	-	trace
Tetradecanal		0.1	-	trace
trans-2-Tetradecenal	(a)	3.7	4.2	trace

(a) Identified for the first time in coriander leaf volatiles

As in the case of peppermint, rosemary, and thyme, the content of six-carbon aldehydes and alcohols increases on picking and drying. Interestingly, a very common hydrocarbon, nonane, has been found for the first time in coriander in a high concentration (15%) in the living leaves. This compound is drastically reduced on picking. Surprisingly, nonane possesses a very characteristic fresh coriander leaf odor. The decanal content also decreases on picking and aging. Decanal is a very orangy chemical and, indeed, it is a character-donating component of orange oil and, thus, lends a citrus note to the living herb.

Contrary to all literature reports on coriander leaf volatiles, the major constituent reported here for the first time is trans-2-decenal, the content of which is relatively constant from living leaf to picked to commercial herb oil. Two other alpha, beta unsaturated aldehydes, trans-2-dodecenal and trans-2-tetradecenal, both reported also for the first time, are present in appreciable quantities in both the living and dried leaf and at lower levels in the oil. A large number of related aldehydes and alcohols, both saturated and unsaturated, many reported for the

first time in coriander, were identified in the living leaf and in the oil but were not found on drying of the leaf.

The headspace volatiles of both living green coriander seed and picked, dried green seed were analyzed as well as commercial seed oil. Even though green coriander seed has quite a different odor from the oil obtained from ripe seed, these aroma profiles are presented in Table IX for the purpose of comparison.

Table IX. Comparative Analysis of the Volatiles of Coriander Seed

Compound	Living Green Seed % (AN)	Picked Dried Green Seed % (AN)	Comm. Seed Oil
alpha Pinene	0.1	0.3	5.1
Linalool	20.9	67.1	73.3
Camphor	0.3	-	4.8
3-Decenal (a)	0.4	-	-
Decanal (a)	3.1	0.1	-
trans-2-Decenal (a)	24.9	-	-
trans-2-Decenol (a)	3.8	-	-
trans-2-Undecenal (a)	3.7	-	-
trans-2-Dodecenal (a)	16.7	-	-
trans-2-Tetradecenal (a)	3.8	-	-
Geranyl acetate	10.0	12.5	2.2

(a) Identified for the first time in coriander seed volatiles

As reported in the literature, linalool constitutes two-thirds of coriander seed oil volatiles. On the other hand, it is only 21% in the living green seed. At the same time, it is drastically increased to 67% on picking and drying of the seed. Geranyl acetate, which is one of the character impact components of coriander seed oil, is present to the extent of 10% in the living seed volatiles but is reduced to 2% in the commercial oil. It is obvious from the table that unsaturated aldehydes and alcohols are major constituents of the living green seed, but these compounds completely disappear after picking and also are absent in the commercial oil. In the opinion of the present authors, the unsaturated compounds shown in Table IX, which have not been reported before as constituents of coriander, are the character donating components of the green seed. They are, undoubtedly, justification for the Greek word "koriannon" for "bug".

It has been demonstrated and proved by ample examples from flowers, fruits, herbs and spices that the volatile constituents of living natural products differ considerably from those of the corresponding picked entities, justifying the assertion that the odor is completely different on picking. To our knowledge, this scientific observation has never been made before. With these data, in IFF we are creating new true-to-nature flavor and fragrance compositions.

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Chapter 15

Flavor of Cooked Meats

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The spectrum of volatile flavor components of cooked meats from different species was investigated. The chemical nature of flavor volatiles was representative of most classes of organic compounds. Hexanal was found to be the predominant volatile component in each case and its content was directly proportional to the amount of TBA-reactive species, while inversely proportional to the flavor acceptability of meats. Nitrite curing depressed the production of lipid oxidation products and nitrite-free curing composition duplicated the action of nitrite on meat, flavorwise.

Flavor is an important sensory aspect of the overall acceptability of meat products. It is perceived as the simultaneous stimulation of our taste and odor senses due to high molecular weight components and volatile chemicals present in cooked meats. The overwhelming effects of flavor volatiles has a tremendous effect on sensory acceptability of foods even before they are consumed.

Meat from different species constitutes an integral part of our diet (except for vegetarians) and provides us with a good source of well-balanced amino acids. Although raw meat has little flavor and only a blood-like taste, it is a rich reservoir of non-volatile compounds with taste tactile properties, as well as flavor enhancers and aroma precursors (1,2). Non-volatile precursors of cooked meat flavor are water-soluble substances and these include amino acids, peptides, reducing sugars and vitamins, particularly vitamin B₁ (thiamine). On cooking, free amino acids (e.g. cysteine/cystine) are produced from the action of proteolytic enzymes which starts during the post-mortem period; breakdown of glycogen results in the production of glucose, fructose, etc. (3,4). These, or their breakdown products, together with other low molecular weight water-soluble matters such as thiamine or its breakdown products react with one another. Often products of one reaction become precursors for others. Thus, interactions of this type and specifically non-

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enzymatic browning or Maillard reactions (5) lead to the formation of a large number of important volatile chemicals which are essential for cooked meat flavor development (6). These together with a contribution from reactions of lipids play an important role in the overall flavor of meat which is distinct from species to species.

Fat portion of meats, particularly their phospholipid components, undergo autoxidation/degradation (7) and produce an overwhelming number of volatiles. Fats also serve as a depot of fat-soluble compounds that volatilize on heating and strongly affect flavor. Since compositional characteristics of lipids in meats, vary from one species to another, these factors may be responsible for the development of some species-specific flavor notes in cooked meats (8,9). Obviously presence of 4-methyloctanoic and 4-methylnonanoic acids with a mutton-specific flavor note is an exception. These branched fatty acids are biosynthesized by sheep (10,11). The swine sex odor compounds, mainly associated with males, are another exception (12).

Presence and concentration of hemoproteins and free iron in meats from different species may also influence the rate of lipid autoxidation/degradation during the cooking and subsequent storage periods (13). Thus, development of off-flavors and unpleasant odors referred to as "warmed-over flavor" (7) depends primarily on the degree of unsaturation of lipid components of meats and somewhat on the level of iron-porphyrin materials present in the muscle.

Cured meats, on the other hand, have a distinct and pleasant aroma which does not change significantly even after prolonged storage at refrigerated temperatures. Antioxidant activity of nitrite, the most important ingredient of the cure, may account for this observation (14,15).

This study was undertaken to unravel some aspects of the flavor of cooked meats and primarily to describe the effect of lipid autoxidation on the flavor of cooked meats.

Meat Flavour Volatiles

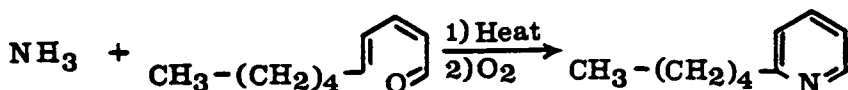
Nearly 1000 compounds have so far been identified in the volatile constituents of meat from beef, chicken, mutton and pork (6). The largest number of volatiles has been determined in beef and these were representative of most classes of organic compounds. Hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids, esters, lactones, ethers, sulfur and halogenated compounds as well as different classes of heterocyclic substances (Figure 1) namely furans, pyridines, pyrazines, pyrroles, oxazol(in)es, thiazol(in)es, thiophenes were present in cooked meat flavor volatiles as shown in Table I. Many of these compounds are unimportant to the flavor of meat and some may have been artifacts (16).

In our opinion, the predominant contribution to flavor seems to come from sulfurous and carbonyl-containing volatiles. While many of the sulfur-containing volatiles are known to have meaty aromas, volatile carbonyl compounds generally are formed by lipid autoxidation/degradation and do not possess meaty flavor notes. However, it has been indicated that the carbonyl compounds are responsible for the "chickeny" aroma of cooked chicken (17). Thus, lipid autoxidation appears to yield the character impact compounds for chicken (18).

TABLE I. Chemical Classes and Numbers of Volatile Constituents of Meats

Class	Beef	Chicken	Mutton	Pork	Cured Pork
Hydrocarbons	123	71	26	45	4
Aldehydes	66	73	41	35	29
Ketones	59	31	23	38	12
Alcohols and Phenols	64	32	14	33	10
Carboxylic acids	20	9	46	5	20
Esters	33	7	5	20	9
Ethers	11	4	-	6	-
Lactones	33	2	14	2	-
Furans	40	13	6	29	5
Pyridines	10	10	16	5	-
Pyrazines	48	21	15	36	-
Pyrroles	4	6	1	9	1
Oxazol(in)es	10	4	-	4	-
Thiazol(in)es	17	18	5	5	-
Thiophenes	37	8	2	11	3
Other nitrogen compounds	6	5	2	6	2
Other sulfur compounds	90	25	10	20	30
Halogenated compounds	6	6	-	4	1
Miscellaneous compounds	5	2	-	1	11
Total	682	347	226	314	137

Although qualitatively many of the flavor volatiles present in meats from different species are similar, there are quantitative differences. In a recent review MacLeod (4) reported that mutton aromas have a high concentration of 3,5-dimethyl-1,2,4-trithiolane and thialdine (2,4,6-trimethylperhydro-1,3,5-dithiazine) and other sulfur compounds due to the presence of a higher concentration of sulfur-containing amino acids in mutton than in beef or pork. Furthermore, it was indicated that mutton aromas contain many alkyl-substituted heterocyclic compounds which may have been formed from the reaction of 2,4-alkadienals with NH_3 produced from thermal degradation of amino acids (19). These compounds are



generally thought to be responsible for the roasted flavor notes and are associated with roasted rather than boiled meats (20). Again higher concentration of amino acids and lower content of sugars in mutton, as compared to beef or pork, accounted for this observation (21). These together with the presence of a larger number of carboxylic acids, and particularly branched chain saturated acids, and high levels of sulfur compounds may account for the rejection of mutton by certain consumers. In beef, mercaptothiophenes and mercaptofurans were significant contributors to its flavor and generally a lower contribution from the lipids on their overall flavor was observed.

Volatiles with Meaty Aromas

A total of 64 sulfur-containing compounds with meaty flavor characteristics have so far been identified in meat volatiles, from which only 7 were acyclic sulfides and thiols (Table II). Most of the sulfurous volatiles of cooked meats are organoleptically active. While at low concentrations present in cooked meats they exhibit a pleasant meaty aroma, at high concentrations their odor is objectionable. These compounds are generally produced from cysteine/cystine, glutathione, and thiamine upon the cooking of meat (Figure 2). Many of the sulfurous volatiles of meat with an active flavor note are heterocyclic in nature (22) and contain one or more sulfur atoms in their ring structure or as a side chain (Table II). A number of sulfur-containing compounds with meaty aromas have also been synthesized (23,24). These were generally thiols of substituted furans and thiophenes. Interestingly, none of these has been found in meat volatiles.

Volatiles with a meaty flavor note generally present in meats from different species are perhaps qualitatively the same, however, their quantities vary from one species to another (25,26). To date, only 13 non-sulfurous volatiles with meaty aromas have been identified in meats and some may indeed be artifacts (Table II).

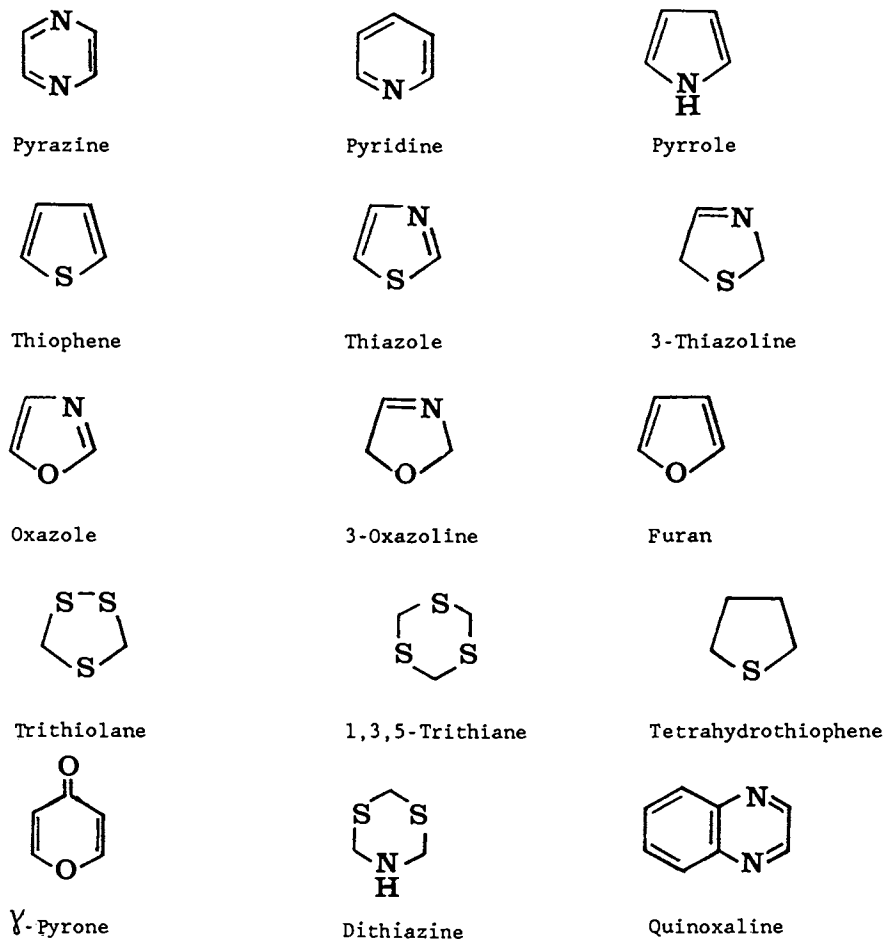


Figure 1. Chemical structures of some heterocyclic flavor volatiles of cooked meats.

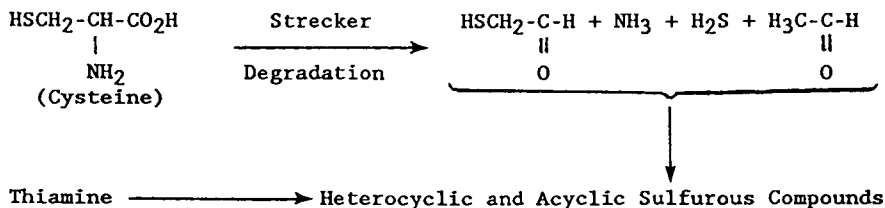


Figure 2. Formation of products from breakdown of cysteine and thiamine.

Impact of Lipid Autoxidation/Degradation on Meat Flavor

The development of oxidative flavors and off-flavors is an important factor in acceptance or rejection of cooked meats. One of the important reactions involved in the formation of volatile compounds in meat, and meat products in general, is the autoxidation of unsaturated fatty acids. Phospholipid components of meats are generally rich in polyunsaturated fatty acids and hence are generally prone to autoxidation (27).

Autoxidation is described as having an initiation, a propagation and a termination step. The susceptibility to autoxidation depends on the ability of fatty acids to donate a hydrogen atom during the propagation step. Thus, the carbon atoms adjacent to double bonds tend to donate a hydrogen atom leading to the formation of resonance-stabilized radicals. The primary products of lipid autoxidation are hydroperoxides and these are odorless in nature. However, upon decomposition of hydroperoxides, secondary products such as hydrocarbons, alcohols, ketones, and aldehydes are produced (Figure 3) and these influence the flavor of meat from different species. Depending on the composition of the fatty acids in lipids, the proportion of these oxidation products vary significantly. Furthermore, such products can themselves undergo further oxidation and decomposition, thus producing a large number of new products which include short-chain hydrocarbons, aldehydes, dialdehydes, epoxides, ketones, acids, alkyltrioxanes, dioxolanes, furans, as well as dimers and polymers.

Although autoxidation of lipids in foods is generally considered as unwanted, certain products of lipid autoxidation at low concentrations are necessary to the characteristic odor and aroma properties of meats from different species (8,9,28). Therefore, the concentration and relative abundance of these chemicals in meat volatiles determine whether they play a desirable or an undesirable role in flavor characteristics of cooked meats. Thus, the origin of flavor and off-flavors developments, which are somewhat species-specific, are perhaps the same. So, in freshly cooked meats the specific flavor of meat which is species-specific develops and progression of autoxidation results in the formation of undesirable warmed-over flavor in cooked meats upon storage.

Aldehydes and ketones, major secondary products of autoxidation are known to impart burnt, sweet, fatty, painty, metallic and rancid flavor notes to meats (6). Many aldehydes also have low odor and flavor thresholds and can be perceived at low concentrations (29, 30).

Malonaldehyde, a major product of autoxidation of polyunsaturated fatty acids is a very reactive substance and reacts with amino acids, proteins and other chemical substances present in meats. Its concentration is generally determined by the 2-thiobarbituric acid (TBA) test. Malonaldehyde may be used as an indicator for evaluation of the oxidative state of cooked meats. It has been reported that warmed-over flavor in beef is generally perceived when TBA number of cooked meats exceed numerical values of 0.5 to 1.0 (31). Malonaldehyde has also been implicated as having mutagenic and perhaps carcinogenic effects (32). Its presence further affects the rheological properties and texture of cooked meat products. Despite these, malonaldehyde has very little or no

TABLE II. Meat Flavor Volatiles with "Meaty" Aroma

Class of Compound	Number ^a	Compound	Example	Flavor note ^a
Sulfides and Thiols, acyclic	7	Mercaptan, methylthioethane	meaty (1-5 ppb), onion	
(Hydro)Furans with sulfur-containing side chain	17	Furfurylthiol, 5-methyl	meaty (0.5-1 ppb), sulfurous (>1 ppb)	
Thiophenes	11	Thiophene-2-methyl-3-thiol	roast meat	
Di and Trithiolanes	5	1,2,4-Trithiolane	roast meat	
Trithianes	3	1,3,5-Trithiane, 2,4,6-trimethyl	meaty	
Thiazol(in)es	14	Thiazole	meaty, nutty, pyridine-like	
Thialdines	4	Thialdine	meaty, roast beef	
Pyrazine-furan sulfides	3	Furfurylthio-2-(3-methyl) pyrazine	cooked meat (<1 ppb), coffee-meaty, pleasant, slightly sulfurous,	
Furans	2	Furan, 2-methyl	sickly	
Oxazol(in)es	2	Oxazole, 2,4,5-trimethyl	boiled beef, nutty, sweet, green	
Ketones	4	Cyclopentanone, 3-methyl	roast beef	
Hydrocarbons	1	n-Octane	meaty	
Miscellaneous	5	Thiophenol, 2-ethyl	meaty, burnt	

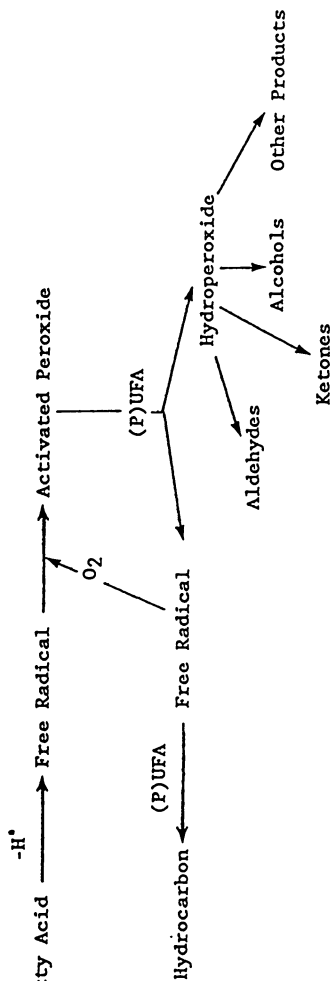
^aRef. 4 and 6.

Figure 3. Mechanisms of lipid autoxidation and formation of products.

odor of its own and, in this respect, may have no effect on the flavor of meat products.

Hexanal, on the other hand, is a predominant breakdown product of lipid peroxidation of ω_6 fatty acids in meats. Its influence on the flavor of cooked meats, especially pork, mutton and chicken is significant. It has been described as having unpleasant, rancid, green and pungent flavor notes (33,34). Its content in cooked ground pork was directly proportional to the amount of TBA-reactive substances (TBARS) present (Figure 4). Relative abundance of some of the other aldehydes with respect to hexanal (arbitrarily set at 100) is given in Table III (unpublished results). Similar results were obtained for cooked ground chicken and mutton; however, beef was somewhat less susceptible to autoxidation, and hexanal was less abundant in the volatiles of beef.

Lipids or lipid breakdown products may also be involved in the formation of 2-alkyl substituted heterocyclic compounds with roast and/or fried flavor notes (19,35). Therefore, lipid-derived volatiles may have a special role in the development of flavor of roasted and or barbecued meats.

Flavor Volatiles of Nitrite-Cured and Nitrite-Free Treated Meats

Nitrite is the unique ingredient of the cure due to its role in the development of color, flavor, as well as oxidative and microbiological stability to meats (36). Each of these properties could be duplicated, however, no single compound has been found with such multifunctional properties. Although nitrite is closely associated with cured-meat aroma (14,15) the chemical changes that are responsible for the unique flavor are not clearly understood (27). A limited number of publications have appeared and a number of volatile chemical constituents have been identified in cured pork. Of particular interest is the work of Cross and Zeigler (37) in which volatile constituents of both cured and uncured ham were examined. These authors reported that the concentration of aldehydes, and especially pentanal and hexanal, was greatly reduced in cured meats. They also found that the volatiles from uncured chicken and beef passed through a solution of 2,4-dinitrophenylhydrazine had an aroma similar to that of cured ham.

Our own work has shown a great decrease in the concentration of the volatiles in the cured, as compared to uncured, meats (Figure 5) (38). The concentration of aldehydes originally present in cooked pork was reduced to $\leq 1\%$ of their original quantities (Table III, unpublished results). However, we did not identify any new flavor active compound which could have been responsible for the cured flavor. Lipid oxidation, as measured by TBA number, was almost eliminated in cooked pork by adding nitrite at a level of 150 ppm (39). Furthermore in preliminary evaluations, our untrained panelists were unable to differentiate amongst the flavor of nitrite-cured meats prepared from beef, chicken, mutton and pork (unpublished results).

It may then be reasonable to postulate that meat on cooking acquires a characteristic species flavor which is caused by the volatile carbonyl compounds formed by oxidation of its lipid components. Due to the strong antioxidant activity of nitrite, however, such oxidation products are either absent or are present

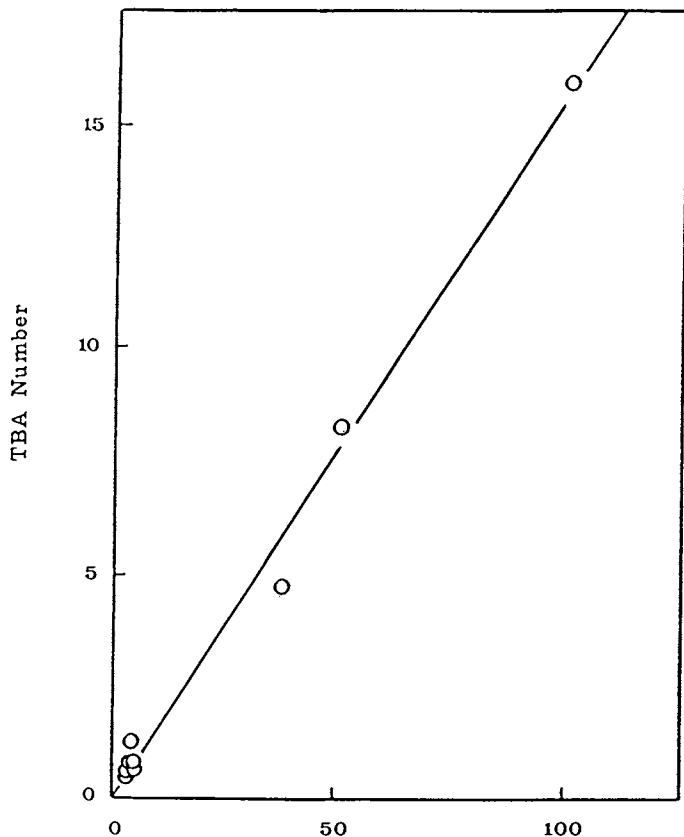


Figure 4. Relationship of TBA numbers (35 days of storage) with relative hexanal content (2 days of storage). (Reproduced with permission from Ref. 42. Copyright 1987. Can. Inst. Food Sci. Technol. J.).

TABLE III: Some Aldehydes and their Relative Abundance in Cooked Ground Pork

Compound	Relative Abundance		Odor Threshold ppb
	Uncured	Cured	
Hexanal	100	7.0	4.5
Pentanal	31.3	0.5	-
Heptanal	3.8	<0.5	3.0
Octanal	3.6	<0.5	0.7
Nonanal	8.8	0.5	1.0
2-Octenal	2.0	-	3.0
2-Nonenal	1.0	-	0.08
2-Decenal	1.1	-	0.3
2-Undecenal	1.4	0.5	-
2,4-Dodecadienal	1.1	-	-

only in minute quantities in cooked cured products. Hence, it may be justified to assume that the flavor of nitrite-cured meats is actually the basic and natural flavor of meat from different species without being influenced by the overtones derived from autoxidation/degradation of their lipid components. However, there is a possibility that cured-meat flavor may have indeed been formed via a mechanism which is unrelated to lipid peroxidation.

In our search for nitrite alternatives, as far as flavor and oxidative stability is concerned, we examined the effect of commonly used adjuncts in meat curing, as well as a large number of antioxidant/sequesterant systems (40-42). In particular, the effect of sodium ascorbate (SA) and sodium tripolyphosphate (STPP) on the oxidative state of cooked meats was studied. These additives lowered the TBA numbers by a factor of about 2 and 4, respectively (Table IV). When used in combination, a strong synergism was observed. Furthermore, an increase in the concentration of SA and/or STPP resulted in a decrease in the TBA values as depicted in Figure 6 (43). Addition of 30 ppm of butylated hydroxyanisole (BHA) or tert-butylhydroquinone (TBHQ) further reduced the TBA numbers and in fact the latter values were even lower than those obtained for meats treated with sodium nitrite (Table IV) (41).

The content of hexanal (Table III) and other lipid oxidation-derived flavor compounds, of meats treated with STPP and/or SA was similarly reduced and the spectrum of flavor volatiles of meats was simplified (Figure 6C), and was similar to that from nitrite-cured meat (Figure 6B). The data for hexanal content are in agreement with Cross and Zeigler's findings (37). Both SA and STPP alone lowered the amount of hexanal in the meat and when used in combination, a strong synergistic effect was observed, thus lending support to our previous findings on TBA values (see above). Although additions of BHA, TBHQ or nitrite, at a level of 30 ppm, had a further effect in reducing the amount of hexanal, the major effect was due to the combination of SA and STPP (42). Furthermore, flavor acceptability of nitrite-free treated samples was not significantly different from that of nitrite-cured meat, as determined by our untrained panelists (Table IV). Therefore, it appears that nitrite may not be an essential ingredient for the development of characteristic flavor of (certain) cured meat products.

Future Research Needs

As a result of the availability of sophisticated instrumentation and separation techniques some remarkable progress has already been made in meat flavor research and this trend is expected to continue. Although a variety of factors are known to affect the development of meat flavor, no single compound/group of compounds, or factor has yet been found that could play the principle role and the true chemical nature of meat flavor, and particularly species differentiation, is not fully understood. Most importantly very little is known about the origin of cured-meat flavor. The curing process seems to simplify the composition of the volatile constituents and eliminates the overtones related to species-specific flavor notes. Thus, work in this area would have a major impact in meat-flavor research and may prove to be extremely

TABLE IV. TBA Numbers, Hexanal Content, and Sensory Scores of Meat ^a

No.	Meat System Additives (ppm) ^b	TBA Number	Hexanal	Sensory Score
1	No Additives	15.93(4.72)	100	2.8
2	(1) + SA(550)	8.30(1.35)	50.1	-
3	(1) + STPP(3000)	4.79(0.21)	38.0	5.1
4	(2) + STPP(3000)	1.33(0.20)	4.1	-
5	(4) + BHA(30)	0.22(0.20)	1.6	5.3
6	(4) + TBHQ(30)	0.27(0.21)	3.0	5.6
7	(4) + NaNO ₂ (30)	0.56(0.30)	2.1	-
8	(4) + NaNO ₂ (150)	0.43(0.25)	2.0	5.7
9	(2) + NaNO ₂ (150)	0.39(0.28)	2.8	-

^aFrom Ref. 42 and unpublished data. Cooked meat were stored at 4°C for 2 days for hexanal, sensory score and TBA numbers (in brackets) determination. Other TBA numbers were determined after 35 days of storage.

^bAll meat samples contained 2% NaCl and 1.5% sucrose. The additives were sodium ascorbate: SA, sodium tripolyphosphate: STPP, butylated hydroxyanisole: BHA, tert-butylhydroquinone: TBHQ, and sodium nitrite, NaNO₂.

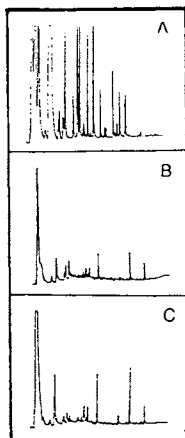


Figure 5. Gas chromatograms of volatiles of meats with A: no additive, B: sodium ascorbate (550 ppm) and sodium nitrite (150 ppm), and C: sodium tripolyphosphate (3000 ppm), sodium ascorbate (550 ppm) and butylated hydroxyanisole (30 ppm).

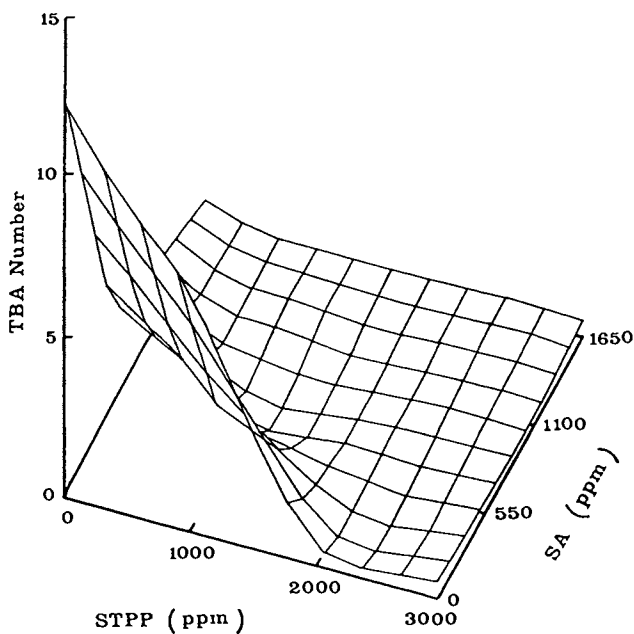


Figure 6. 3-D plot showing the effect of sodium tripolyphosphate (STPP) and sodium ascorbate (SA) on the TBA numbers of cooked pork after 4 weeks of storage at 4°C. (Reproduced with permission from Ref. 43. Copyright 1987. Can. Inst. Food Sci. Technol. J.).

fruitful. Identification of possible flavor-active components present in extremely low concentrations may be rewarding. Use of non-degradative methods of isolation such as super-critical extraction and identification of flavor compounds by HPCL and HPLC-MS methods are highly recommended.

Furthermore, growing needs for creation of novel fast foods with characteristic aromas, consumers awareness and their desire for natural flavors may dictate, in part, the future direction that meat flavor research may pursue. Creation of simulated meat flavors with acceptable quality characteristics may satisfy a growing and discriminating population of health-conscious consumers. The role of lipids and lipid-derived aroma volatiles and creation of species-specific meat flavors deserves further investigation.

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Chapter 16

New Trends in Black Truffle Aroma Analysis

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The volatile compounds in the atmosphere of cold stored Black Perigord Truffles (Tuber Melanosporum) were adsorbed onto a Tenax trap by means of a vacuum pump. The efficiency of the sampling method was sensorially validated. The volatiles eluted from the trap by heat desorption were analysed by capillary gas chromatography - mass spectrometry. A total of 26 compounds was identified. Their contribution to the final aroma impression was discussed.

The isolation of volatile flavor compounds from foods represents a major problem in analytical studies of food flavor. Headspace methods are especially attractive since they are rapid, simple and measure what is typically presented to the nose.

We have previously used a Dynamic Headspace method, optimized with Experimental Design (1), for the isolation of the volatiles from Black Perigord Truffles (Tuber Melanosporum). Truffles are underground mushrooms that grow in symbiosis with certain trees, especially oaks. One finds them in several regions of Europe, particularly in France, where their flavor is very much appreciated by gourmets. Our studies, carried out on truffle flesh (2,3), and entire truffles (Talou, T. et al, J. Sci Food Agric., in press), allowed us to identify the major volatile compounds. Due to the low sampling weight, however, the method appeared to be ineffective for isolating compounds extremely low in concentration.

The atmosphere of cold stored Black Truffles is particularly rich in volatile compounds which impart the truffle aroma. We therefore developed a modified gas headspace sampling procedure for their isolation.

The aims of this study were to evaluate the efficiency of our sampling method for trapping volatile components important to the aroma of Black Perigord Truffles, and the analysis of minor volatiles via their identification with capillary gas chromatography-mass spectrometry.

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Experimental

Plant material Fresh Black Perigord Truffles (Tuber Melanosporum) for analysis were purchased by Pebeyre Ltd., a company specializing in truffle marketing. Collected essentially in the South East of France, they were fully ripe (4) and released their characteristic aroma. Received the day after gathering in wicker-baskets, 3 truffles were hand-picked and then stocked in a cold storage of 15 m³.

Volatile collection was generally begun the day after receiving the packages. 1000 kg of unbrushed truffles were in storage when atmosphere capture was carried out. Three isolations were carried out in duplicate during February 1987.

Preparation of Tenax tubes and Blank Tenax chromatogram Tenax GC (60-80 mesh) was conditioned by heating 10 g at 250°C for 24 h in a dry Helium flow of 50 mL/min. Conditioned Tenax (0,2 g) was packed into a 6 cm long steel tubes (6 mm o.d., 4 mm i.d.) and plugged with glass wool. A blank Tenax gas chromatogram was obtained by heat desorbing the tube into a gas chromatograph (GC) column using the procedure described under Heat Desorption of Aroma volatiles section.

Additional cleaning of Tenax tubes was standardized by heat desorption at 200°C for 30 min in the oven of the D.C.I. System.

Headspace sampling method An idealized diagram of the sampling system is shown in Figure 1. It consisted of a vacuum pump, a needle valve, a flow meter and 2 auxiliary Tenax tubes. Connections were made by Swagelock Teflon unions. Cold storage atmosphere was passed through the two "in-serie" ambient Tenax tubes by applying suction to the outlet of the tube by means of the vacuum pump.

Preliminary experiments, the results of which are summarized briefly under Results and Discussion, led to the following optimized sampling method. Volatile collection was carried out for 30 min at the cold storage temperature (4°C) and with a vacuum pump flow rate of 50 mL/min. Then, the sampled Tenax tubes were removed, capped and ready for GC analysis the same day.

Heat Desorption of Aroma volatiles The gas chromatographic method employed a D.C.I. System (Desorption - Concentration - GC Introduction), available from Delsi Instruments (5). A schematic diagram of this apparatus is given in Figure 2.

One of the auxiliary Tenax tubes (1) was placed inside the desorption oven (2), located upstream of the fixed Tenax trap (3) (0,2 g Tenax GC, 60-80 mesh, packed into a 7 cm by 2 mm i.d. stainless steel tube). At controlled temperature (100°C) and low pressure (1,5 psi) the oven was flushed by a 25 mL/min flow of Helium for 10 min. Desorbed and diluted in scavenging gas (a), the volatiles were then concentrated and trapped in the Tenax trap, cooled to -30°C by circulation of liquid nitrogen. By switching a rotary valve (4), carrier gas (b) flowed through the trap (backflush) and towards the GC column (6). By rapid thermal desorption at 240°C, aroma volatiles were directly transferred onto the GC column. Figure 3 shows the chronological sequence for cryogenic volatile adsorption and thermal desorption, and chromatographic separation.

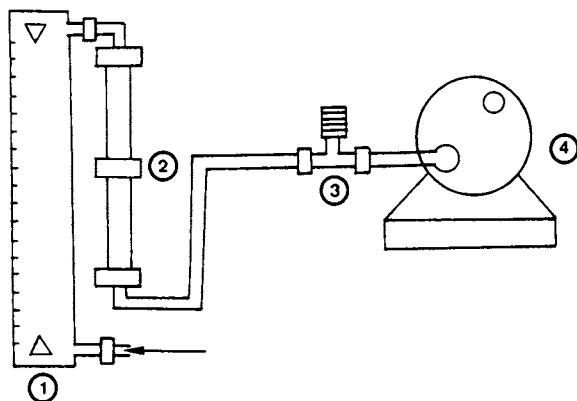


Figure 1. Schematic diagram of the sampling apparatus
1) flowmeter; 2) auxiliary Tenax tube; 3) needle valve; 4) vacuum pump.

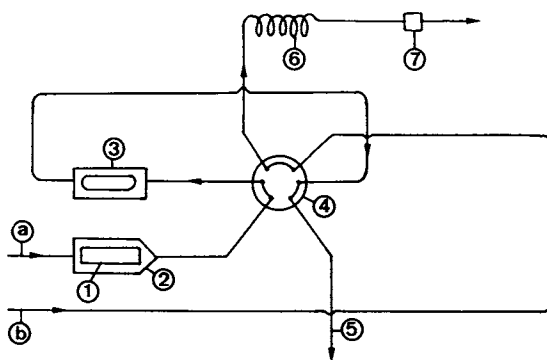
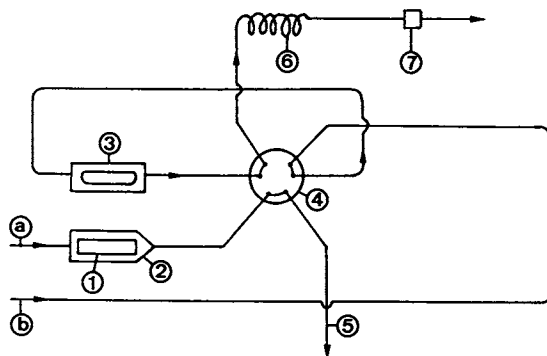
**DESORPTION - CONCENTRATION****INTRODUCTION**

Figure 2. Schematic diagram of the D.C.I. system

a) scavenger gas; b) carrier gas

1) auxiliary Tenax tube; 2) desorption oven; 3) fixed Tenax trap;

4) switching valve; 5) gas vent; 6) GC column; 7) mass spectrometer

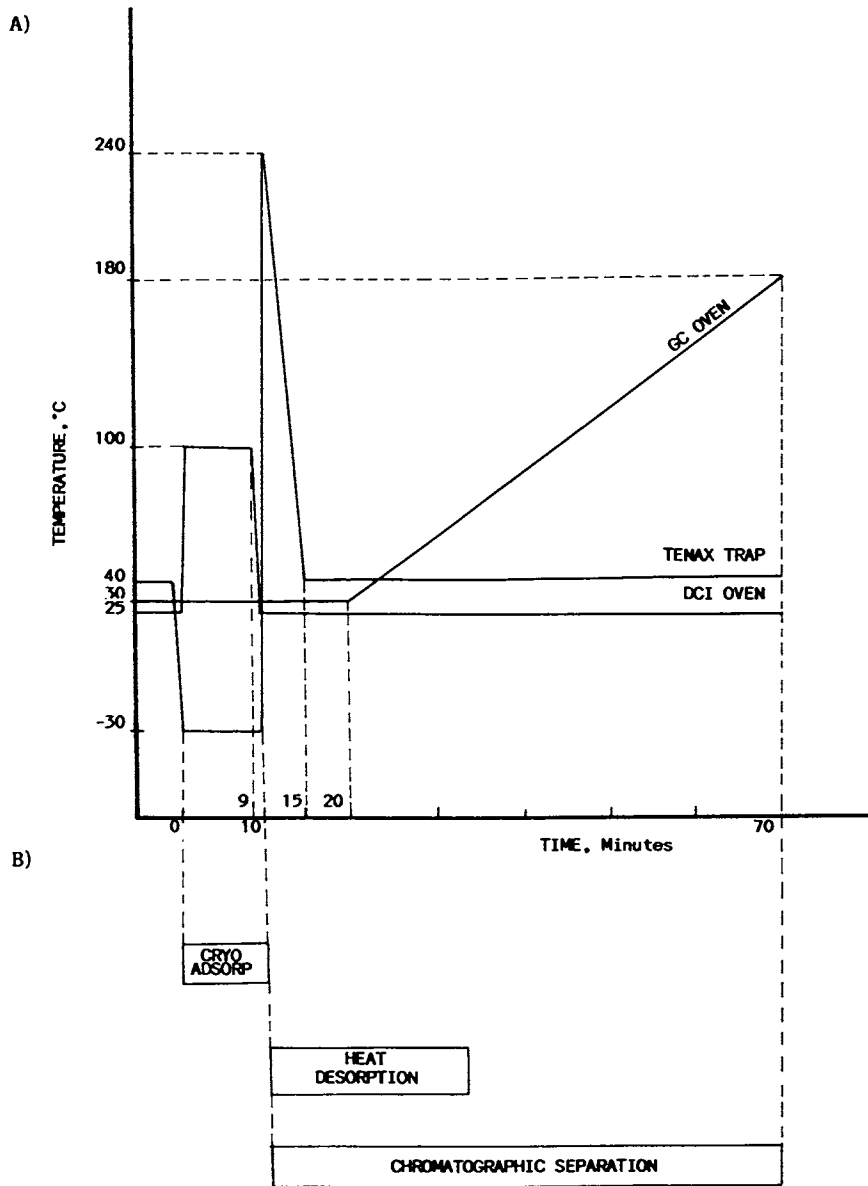


Figure 3. Chronological sequences of GC analysis
 A) temperature sequence; B) time sequence.

Combined Capillary Gas Chromatography - Mass Spectrometry A GIRDEL 31 gas chromatograph equipped with a D.C.I. System was coupled by means of a glass lined tubing interface to a NERMAG R10-10B quadrupole mass filter spectrometer. The system was connected on-line to a dedicated data processing system consisting of a Digital Equipment Co. PDP8 Computer, using SIDAR software including the library of mass spectral data NIH/EPA (6).

The capillary column used was a 50 m X 0,32 mm (i.d.) fused silica UCON 75H 90000 wall coated column. The column oven temperature was programmed from 30 to 180°C at a rate of 3°C/min with a 10-min post injection hold and a 10-min hold at a final limit. Column inlet pressure of Helium was 7 psi and the split 30 mL/min.

Significant Mass Spectrometry operational parameters were as follows : ionization voltage, 70 eV; ionization current, 200 uA; source temperature, 200°C; electron multiplier tension, 1,9 kV; integration time, 1 ms/u.m.a.. For optimum sample transfer, an interface temperature of 200°C was adopted.

Reagents Helium was purified by passage through charcoal, molecular sieve and an Oxisorb trap. Tenax adsorbant was obtained from Alltech Assoc. Inc. Chemical compounds for comparisons of mass spectra and GC retention times were obtained from commercial sources (Aldrich). Sec-butyl and isopropyl formates were synthesized according to Dimicky (7).

Sensory Validation of Sampling and GC Techniques The sensory evaluation was carried out by a panel of three judges (employees of Pebeyre Ltd.). For this study, an external odor port was attached to the gas vent (5) of the D.C.I. System and the rotary valve (4) was not switched (analysis using the Desorption - Concentration mode). Thus, after the thermal desorption of the volatiles from the trap, the rotary valve was positioned so that the unresolved aroma isolate went to our sniffing port. The response was measured as similarity or dissimilarity to Black Truffle aroma.

Sensory Evaluation of the contribution of identified compounds to the aroma In order to identify those compounds mainly contributing to the characteristic flavor of the Black Truffle, the odor of the individual components of the headspace analysis were tested by a panel of eight judges (trained in sensory evaluation of truffles). The compounds tested were diluted in vegetable oil, in a range of concentrations from 30 to 300 ppm. 5 point scales (5 = exceptionally good full truffle aroma, 1 = not different from solvent) were used for flavor imitation and intensity.

Results and Discussions

Optimization of the sampling and analytical techniques Preliminary trials were used to determine the optimal adsorbent. Tenax GC was chosen due to its high affinity for organic compounds which it adsorbs reversibly, and its relatively hydrophobic character. This point is important in view of the large volume of water vapor present in the cold storage atmosphere. For this same reason,

subambient traps appeared to be ineffective due to the risk of physical blockage of the Tenax by solidification of atmospheric water vapor.

The optimization of the sampling conditions was necessary to avoid breakthrough from the Tenax, i.e. elution of volatiles partially through the trap during atmosphere capture. Indeed, sampling efficiency decreased with sampling time, breakthrough then occurring, but this was not significantly decreased by using lower sampling flow rates. Under our optimized sampling conditions, the heat desorption of the second Tenax trap which was nearest to the vacuum pump in the sampling system gave a blank chromatogram, showing that there were no losses of volatiles from the first trap during the capture.

The conditions of chromatographic analysis (desorption of volatiles from the Tenax tube and their adsorption on the Fixed Tenax trap) have also been optimized in order i) to obtain a total desorption of volatiles trapped on the auxiliary tube (confirmed by a blank chromatogram for a second heat desorption), ii) to avoid losses of volatiles during the adsorption phase (sensorially verified at the odor port).

One particular volatile isolate desorbed and assessed sensorially at our odor port was described as typical of Black Truffle, showing that the Tenax had adsorbed and desorbed (under the analytical conditions used) volatile components that impart Black Truffle aroma.

The aroma isolation method and heat desorption techniques were therefore validated.

Identification of volatiles compounds A typical total ion current chromatogram of the Tenax trapped Black Truffle (Tuber Melanosporum) volatiles is shown in Figure 4.

The compounds identified by GC-MS are listed in Table I, in order of elution from the GC column with their observed characteristic mass spectral data. The identification of these compounds is based on comparison of their respective mass spectra obtained with those stored in the NIH/EPA library and then with those of authentic compounds. Moreover, an additional search of published standard mass spectra to confirm the identity of unknown was undertaken (8).

The isolation of the major volatile compounds, i.e. low boiling point alcohols, and their relative aldehydes, 2 ketones and one major sulfur compound was consistent with previous reports (2,3). Although alcohols remained the major constituents of headspace volatiles, dimethyl sulfide was present in greater proportion than in our earlier studies.

The similarity of the chromatographic profiles of headspace volatiles of brushed (1,2) and unbrushed truffles (obtained in this study) allowed us to give scientific support to the informal subjective observation that unbrushed and brushed truffle aroma are not significantly different.

Nevertheless, we noticed that the relative concentration of alcohols increased during the period of cold storage. But the experts (employees of Pebeyre Ltd.) reported that the aroma of the analysed truffles were not really altered by this modification of

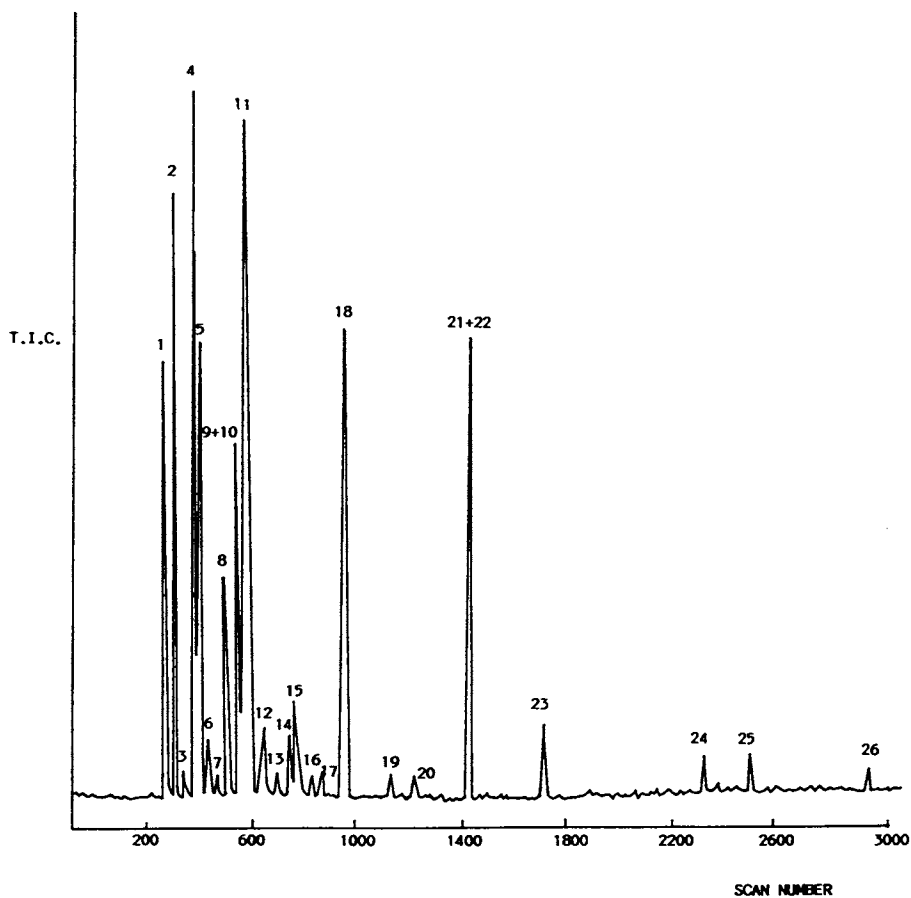


Figure 4. Reconstructed capillary GC-total ion current chromatogram of the Tenax trapped Black Truffle (*Tuber Melanosporum*) headspace volatiles.

Table I. Volatiles identified in atmosphere of cold storage for Black Truffles (*Tuber Melanosporum*)

peak number (a)	compound	mass spectral data (b)	identification GC, MS
1	acetaldehyde	29,44,43,42	GC, MS
2	dimethyl sulfide	42,62,45,46	GC, MS
3	propanal	29,28,27,58	GC, MS
4	acetone	43,58,42,39	GC, MS
5	2-methylpropanal	43,41,72,27	GC, MS
6	isopropyl formate	45,42,43,73	GC, MS
7	ethyl acetate	43,29,61,45	MS
8	2-butanone	43,72,29,27	GC, MS
9	2-methylbutanal	41,57,29,58	GC, MS
10	3-methylbutanal	44,41,43,29	GC, MS
11	ethanol	31,45,46,29	GC, MS
12	sec-butyl formate	45,41,56,29	GC, MS
13	chloroform	83,85,47,48	MS
14	2-butanol	45,31,59,29	GC, MS
15	1-propanol	31,29,42,27	GC, MS
16	toluene	91,92,65,51	MS
17	dimethyl disulfide	94,45,79,46	MS
18	2-methyl-1-propanol	43,33,42,41	GC, MS
19	ethyl benzene	91,106,51,28	MS
20	xylene	91,106,105,77	MS
21	2-methyl-1-butanol	41,29,57,56	GC, MS
22	3-methyl-1-butanol	41,29,42,55	GC, MS
23	anisole	108,78,65,39	GC, MS
24	acetic acid	45,43,60,28	MS
25	benzaldehyde	105,106,77,51	GC, MS
26	2-formyl thiophene	111,112,39,45	MS

a) The peak numbers correspond to numbers in Figure 4; b) The four most intense peaks are reported.

concentrations. This result showed that a short period of cold storage did not affect the organoleptic quality of truffles.

The major advantage of the sampling technique developed, was that some trace chemicals could be trapped and described for the first time as Black Truffle aroma constituents. In particular, some compounds, important flavor contributors, generally appearing in small concentrations, such as benzaldehyde, propanal, ethyl acetate, anisole or dimethyl disulfide - previously identified in Shiitake mushrooms (9) - could be characterized. This was also the case for three aromatic compounds, toluene, xylene and ethyl benzene, well known as raw vegetable constituents (10). In addition, two aliphatic esters, isopropyl and sec-butyl formates, and one cyclic sulfur compound (2-formyl thiophene) previously reported respectively in plums and apples (10) and in coffee and bread products (11) were identified.

Chloroform was certainly an artifact due to treatment of the soil where the truffles were harvested.

Relative odor contribution of components Considered individually, none of the compounds identified duplicated the flavor of the Black Truffle. However, according to the judges, dimethyl sulfide was determined to have a great importance to the final aroma impression. In the same way, 2-butanone and anisole were found to be responsible for an off-flavor characteristic of over-mature truffles.

When mixtures of two and/or three compounds were tested, the panel scores remained low except for the mixture of dimethyl sulfide/2-methylbutanal.

A synergistic effect, which has been defined by Schutte (12) as being due to two or more compounds combining together to give a flavor sensation very different from that of any single component, seemed evident. This was confirmed by the high score obtained by a mixture of all compounds. This oil solution was judged to be very similar to fresh black truffle aroma by the panel (Talou, T. Ph.D. Thesis, in preparation): this indicated that the typical flavor for black Perigord Truffle was the result of an integrated response to the contribution of all the identified compounds.

An industrial application of this result was the elaboration of the first Nature-Identical Black Truffle aromatizer (13). This product was tested with success during one year by well known french cooks in their restaurant. Culinary tests confirm the good organoleptic quality of the flavored products obtained, both for cold or hot dishes.

Conclusions

The headspace sampling technique developed in the present study to collect volatiles from cold stored Black Truffles performed adequately. Indeed, the aroma isolate obtained was described as typical, and 11 minor compounds could be described for the first time as Black Truffle aroma constituents. Moreover, these results allowed the formulation of the first Nature-Identical Black Truffle aromatizer.

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Chapter 17

Fresh Tomato Volatiles

Composition and Sensory Studies

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Analysis of the vacuum volatile constituents of fresh tomatoes was carried out using capillary GLC-MS and packed column GLC separation with infrared, NMR and CI-MS analysis. Evidence was obtained for the presence of the unusual components β -damascenone, 1-nitro-2-phenylethane, 1-nitro-3-methylbutane, β -cyclocitral and epoxy- β -ionone. A method for the quantitative analysis of the volatile aroma components in fresh tomato has been improved and applied to fresh tomato samples. The quantitative data obtained have been combined with odor threshold data to calculate odor unit values (ratio of concentration / threshold) for 30 major tomato components. These calculations indicate that the major contributors to fresh tomato aroma include (Z)-3-hexenal, β -ionone, hexanal, β -damascenone, 1-penten-3-one, 3-methylbutanal, (E)-2-hexenal, 2-isobutylthiazole, 1-nitrophenylethane and (E)-2-heptenal.

The authors are carrying out a continuing study to try to obtain a better chemical definition of fresh tomato flavor and aroma. Studies to develop and apply quantitative methods to the analysis of fresh tomato volatiles have been recently carried out by some of the authors (1,2). Besides the known major compounds a number of compounds were detected in the gas liquid chromatography (GLC) analysis which had spectral data unlike that of any of the 400 compounds previously reported as tomato volatiles (cf. 3). As these compounds occurred in reasonable amounts in fresh tomato it seemed necessary to determine their identities in order to give a satisfactory quantitative picture of fresh tomato volatiles. It also seemed desirable to determine the odor threshold of these compounds to have a better understanding of their probable contribution to tomato aroma.

EXPERIMENTAL

Materials. Tomatoes were grown on experimental and commercial fields near Davis, California during the summer of 1987. Tomato

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breeding lines used included E6203, FM785, GS-12 (Goldsmith-12), Lassen, XPH5498 and others. Freshly picked vine ripe tomato samples were stored at room temperature under normal lighting and used within 3-5 days.

Freshly distilled diethyl ether and saturated CaCl_2 solution were prepared as previously described (2).

Isolation of Volatiles from Tomato Condensate. Condensate from commercial tomato paste production using vacuum concentration was stored at 5°C in the dark and used within a few days. Extraction was carried out with a laboratory built 40 liter liquid-liquid pyrex extraction apparatus. The tomato condensate was first extracted with pentane for 24 hours and then with diethyl ether for 24 hours. The solvent was removed by distillation from a warm water bath using a Vigreux distillation column. The concentrates from a number of batches of tomato condensate were combined. Ethyl antioxidant 330 (1,3,5-trimethyl-2,4,6-tris-[3,5-ditertbutyl-4-hydroxybenzyl]-benzene; ca. 10 ppm) was added and the concentrate stored at -20°C .

Packed Column GLC Separation of Components. The concentrate from above was first separated into two main fractions by micro-distillation. These were Fraction A, b.p. $25-38^\circ\text{C}$ at 0.1 mm Hg (88% of original concentrate) and Fraction B, b.p. $> 38^\circ\text{C}$ at 0.1 mm Hg (residue, 12% of concentrate).

Components were isolated from these distillation fractions using consecutive GLC separation first on a 10 m x 1.3 cm o.d. aluminum column packed with 60-80 mesh Chromosorb G coated with 5% Silicone SF96(50) followed by further resolution on a 3 m x 0.95 cm o.d. Pyrex glass column packed with 60-80 mesh Chromosorb G coated with 1% Carbowax 20-M. A specially designed glass collector packed with Pyrex glass wool was used for the 10 m column (the trap was centrifuged to separate the component from the glass wool) and 10 cm long by 3 mm o.d. pyrex tube collectors for the 3 m column. During collection the traps were cooled with dry ice. The collected samples were sealed in the 3 mm tubes and stored at -20°C .

Infrared and NMR Spectra. Infrared spectra were measured as thin films using ultramicro salt plates with a Perkin Elmer Model 197 instrument. Proton nuclear magnetic resonance ($^1\text{H NMR}$) spectra were measured as solutions in CDCl_3 using a Nicolet NTC 200FT spectrometer.

Gas-Chromatography Mass Spectral (GC-MS) Analyses. Several different studies were carried out. The main study was done using a Finnigan MAT 4500 series quadrupole mass spectrometer and a 60 m x 0.32 mm i.d. DB-1 bonded fused silica capillary GLC column. The column was programmed from $25-250^\circ\text{C}$ at 4° per minute with an inlet pressure of 14 psi. Chemical ionization (CI) mass spectra on some of the components were also obtained using a VG Micromass 70/70 mass spectrometer with isobutane as the reactant gas.

Isolation of Volatiles for Quantitative Studies. The method used was essentially the same as that described previously (2). The whole tomato sample (100g at 25°C) of pieces cut from 3-4 different tomatoes was blended for 30 seconds (using a Waring blender with blades rotating at 13670 rev/min). The mixture was allowed to stand at room temperature for 180 seconds longer and then saturated CaCl₂ solution (100ml) added and the mixture blended for 10 seconds. A standard solution (5.0 ml) containing 20.0 ppm 2-octanone, 20.0 ppm 3-pentanone and 5.0 ppm anethole in water (the standard solution was stored at 5°C in the dark) was then added and the mixture blended again for 10 seconds. The resultant mixture was then poured into a 1 L flask containing an efficient magnetic stirrer. Purified air (3L/minute) was then led into the flask and passed over the vigorously stirred mixture (at 25°C) and out of the flask through a Tenax trap (14 cm long by 2.2 cm i.d.; 10 g). All connections were either Pyrex glass or Teflon. The isolation was carried out for 60 minutes and the trap removed and eluted with 100 ml of diethyl ether. The ether extract was concentrated to ca. 50 µl using a warm water bath and Vigreux distillation column. The Tenax trap was reactivated by passing a stream of purified nitrogen through it at 200°C for 1 hour.

Authentic Samples. Authentic samples of identified compounds were obtained from reliable commercial sources or synthesized by established methods. All samples were purified by GLC separation and their identities verified by mass or infrared spectrometry. 1-Nitro-3-methylbutane and 1-nitro-2-phenylethane were synthesized according to the method of Kornblum et al. (4) by the reactions of 1-bromo-3-methylbutane and 2-phenylethylbromide with sodium nitrite in dimethylformamide and urea.

Odor Threshold Determinations. These were carried out on samples purified by gas chromatographic separation using methods previously described (1) with a panel of 16 to 20 judges.

RESULTS AND DISCUSSION

Three main approaches were applied to fresh tomatoes. The first approach was a qualitative one. It was aimed at the further identification of important aroma compounds. The second approach was designed to develop better methods for the quantitative analysis of important tomato aroma compounds and to apply the methods to various samples of tomatoes. The third approach involved the sensory evaluation of identified tomato volatiles to determine their probable importance to fresh tomato aroma.

Qualitative Approach. Aqueous condensate was obtained from commercial tomato processors from the vacuum (ca. 100mm) concentration of fresh tomato to give tomato paste. The volatile components from this condensate were obtained by continuous liquid liquid extraction using first pentane and then diethyl ether. Enough condensate was processed to give several grams of volatile tomato concentrate. The volatiles obtained from this extraction

showed little evidence of thermally produced Maillard type volatiles and even showed relatively high concentrations of (Z)-3-hexenal which the authors found difficult to isolate quantitatively by their method of vacuum steam distillation in the laboratory. Comparison to volatiles isolated from fresh tomatoes in the laboratory, both by vacuum steam distillation-continuous extraction and by Tenax trapping, showed that they were similar qualitatively although there was considerable quantitative differences.

The volatile concentrate from the commercial condensate was first separated into 2 main fractions by micro distillation under reduced pressure (0.1mm Hg). The distillation fractions were then resolved into their components by packed column GLC separation first with a 10 m Silicone SF96 packed column with further GLC resolution of the Silicone GLC fractions using a 3 m Carbowax 20-M column. Infrared absorption spectra were measured with the separated components. In some cases HNMR spectra and chemical ionization (C.I.) mass spectra were obtained. This additional spectral data was particularly useful for the identification of some unusual compounds, i.e., β -damascenone, 1-nitro-2-phenylethane, 1-nitro-3-methylbutane, β -cyclocitral and epoxy- β -ionone.

The nitro compounds, 1-nitro-3-methylbutane and 1-nitro-2-phenylethane, were particularly difficult to identify because they give very weak parent ions with electron ionization (E.I.). However, C.I. mass spectra gave adequate M+1 ions. Figures 1 and 2 show the mass spectra (E.I.) of these compounds. High resolution mass spectra also gave their empirical formula. They were readily synthesized by reaction of 3-methylbutyl bromide or phenylethylbromide with sodium nitrite. The identification of 1-nitro-3-methylbutane in tomato had been reported previously by Wobben et al. (5) although they had not published any GLC or mass spectral data. None of the other numerous studies of tomato volatiles (cf. 3) had reported finding this compound. It is a relatively prominent component of fresh tomato occurring at a concentration as much as 200 ppb in some varieties such as Ace and related varieties but in other varieties it occurs at lower levels (10-50 ppb). However, it does not seem to be important to fresh tomato aroma because it is a relatively weak odorant with an odor threshold of 150 ppb.

1-Nitro-3-methylbutane bears an interesting relationship to two other known volatile components of fresh tomatoes, isobutyl cyanide and 2-isobutylthiazole and to the non volatile amino acid leucine. This relationship is shown in Figure 3. They all show a similar skeletal arrangement of carbon atoms to the left of the nitrogen.

1-Nitro-2-phenylethane bears a similar relationship to phenylalanine and phenylacetonitrile. The nitro compounds are possibly formed by oxidation of these amino acids. Stone et al., (6) had previously presented evidence (using radioactive isotopes) that leucine was one of the precursors of 2-isobutylthiazole. 1-Nitro-2-phenylethane is a moderately potent odorant with an odor threshold of 2 ppb and as later discussed probably contributes to the tomato aroma.

β -Damascenone had not been previously reported in tomatoes until

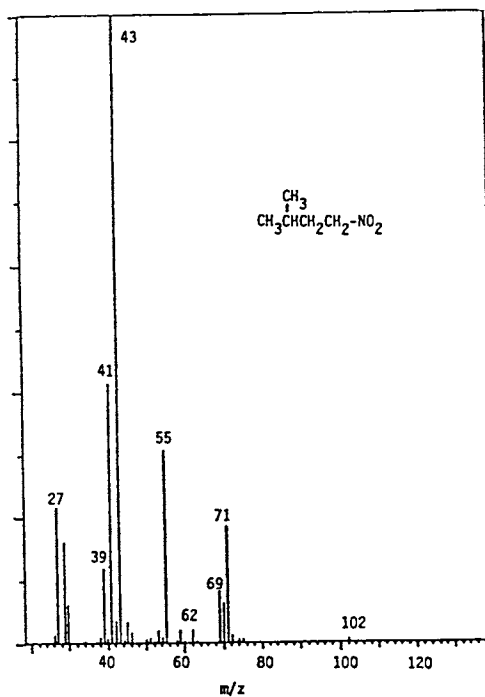


Figure 1. Mass spectrum (E.I.) of 1-nitro-3-methylbutane.

recently by some of the authors (7). Its presence had been well established in a number of other products such as apples (8) and grapes (9). It was identified by the authors in tomatoes by comparison of its mass spectrum, capillary GLC retention data and infrared absorption spectrum with those of an authentic sample. In reviewing mass spectral data on tomato volatiles they had recorded in 1970 (10) some of the authors found that they had obtained a mass spectrum of β -damascenone then but had not been able to identify it. In that year the structure of β -damascenone had only just been elucidated and proved by synthesis by Demole et al., (11). β -Damascenone has been shown to be a potent odorant (9,12) which was verified by the authors who determined its odor threshold to be 0.002 parts per billion (ppb) of water. Its concentration in fresh tomatoes was found to be 1-3 ppb (500-1500 times its threshold) so that it seems very likely that β -damascenone is an important aroma component of fresh tomatoes.

β -Cyclocitral had not been previously reported in tomatoes except by the authors (2). Structurally related to β -ionone it probably also results from oxidative degradation of β -carotene. With an odor threshold of 5 ppb it is a moderately potent odorant. However, its concentration in blended tomatoes is usually below this concentration and it seems unlikely that it can make a significant contribution to fresh tomato aroma.

Epoxy- β -ionone had been reported previously by Viani et al., (13), Schreier et al., (14) and Wobben et al., (5). In the present study besides the mass spectrum an infrared absorption spectrum was also obtained and was found to be identical to that of an authentic sample. An odor threshold was determined in water solution to be 100 ppb. It is, therefore, a relatively weak odorant and as its concentration, in all fresh tomato samples examined, is well below this figure it seems unlikely that it can contribute to fresh tomato aroma.

Quantitative Studies. A method for the quantitative analysis of fresh tomato volatiles using a saturated CaCl_2 solution to deactivate tomato enzymes and Tenax trapping had been developed by the authors (1,2). The method also included the use of the internal standards 3-pentanone, 2-octanone and anethole added (as a dilute solution in water) just after the addition of the saturated CaCl_2 . The authors had shown (2) that for a 64% recovery of a compound a simple equation

$$V_a = V_w / K$$

(where V_a = volume of sweep gas, V_w = volume of aqueous solution and K = air/water partition coefficient) gave the amount of sweep gas needed for Tenax trapping. There are some other factors involved also, of course, such as adsorption of volatiles on the glass walls of the flask and sweep gas outlet head. From some studies that the authors have carried out, this adsorption is probably negligible for most compounds with boiling points less than about 2-octanone but become more important with higher boiling compounds such as eugenol. As with adsorption on Tenax, adsorbed compounds are also desorbed from the glass surface by the continual flow of sweep gas over the surface. The greater the amount of sweep gas passed over the surface, the smaller will be the amount of compound remaining on the glass surface.

The 1 hour trapping period is practical for typical quantitative studies where many samples are involved. It would be desirable to shorten this time even more. As can be seen from the above equation this might be accomplished by decreasing the volume of sample V_w which in turn decreases the required volume of air, V_a . Thus halving V_w halves V_a (this ignores glass surface adsorption effects) to give a 1/2 hour trapping period. However, a smaller amount of sample would be available for GLC analysis which makes handling more difficult and sensitivity borderline for the lowest concentration components (such as β -ionone).

Studies with model systems of standard solutions of components in water using the same isolation procedure outlined by the authors for the tomato (2) showed satisfactory recoveries of most tomato volatiles. A few compounds gave unacceptable recoveries for the 1 hour sweeping period used. These were 2-phenylethanol which gave a 3% recovery (relative to anethole) and eugenol which gave a 0.5% recovery (relative to anethole) for the 1 hour sweep period. With 24 hour sweep periods both of these compounds gave better than 50% recoveries.

Experiments were also carried to determine the recovery of known amounts of tomato compounds added to samples of macerated tomato that had been previously heated to boiling and which contained very little of the fresh volatiles tested. The recovery obtained from the macerated tomato relative to the internal standards was (within ca. $\pm 10\%$) the same as that obtained for water solutions.

Comparison of the concentrations of the different components of the tomato was made previously (2) for the separated main parts of the tomato i.e. the skin, pulp, fluid and seed. These studies had shown that the skin and pulp contained the highest concentrations of volatile components and that the seeds contained essentially none.

Concentrations found for a vine ripe (macerated) common commercial tomato line (GS-12) are shown in the first column of Table I. The data are the average of figures from three separate isolations.

Sensory Approach. The main sensory studies applied have been in the determination of odor thresholds of components and the calculation of odor unit values (U_o), the ratio of the concentration of the component in the food to its odor threshold in water. The results of these studies are summarized in Table I. It can be seen that (Z)-3-hexanal shows the most odor units followed by β -ionone, hexanal, β -damascenone, 1-penten-3-one, 3-methylbutanal, (E)-2-hexenal, 2-isobutylthiazole, 1-nitro-2-phenylethane and (E)-2-heptenal. β -Damascenone and 1-nitro-2-phenylethane are new members of this group.

Odor descriptions of dilute water solutions of β -damascenone and 1-nitro-2-phenylethane were also obtained using a panel of 18-20 judges. β -Damascenone as a 10 ppb solution in water was described as having an odor most similar to (1) prunes (2) apple (3) sweet character and (4) tomato in that order (i.e. being most like prunes). The odor of 1 ppm solutions of 1-nitro-2-phenylethane were described as (1) green (2) geranium (3) tomato and (4) oily in that order.

Table I. Concentrations of major volatile fresh tomato components using blending procedure, odor thresholds in water solution and Log Odor Units. Compounds listed in descending order of their Log odor units

Compound	Conc. ppb	Odor Thresh. ppb in H ₂ O	Log. Odor Units
(Z)-3-hexenal	12000	0.25	4.7
β-ionone	4	0.007	2.8
hexanal	3100	4.5	2.8
β-damascenone	1	0.002	2.7
1-penten-3-one	520	1	2.7
3-methylbutanal	27	0.2	2.1
(E)-2-hexenal	270	17	1.2
2-isobutylthiazole	36	3.5	1.0
1-nitro-2-phenylethane	17	2	0.9
(E)-2-heptenal	60	13	0.7
phenylacetaldehyde	15	4	0.6
6-methyl-5-hepten-2-one	130	50	0.4
(Z)-3-hexenol	150	70	0.3
2-phenylethanol	1900	1000	0.3
3-methylbutanol	380	250	0.2
methyl salicylate	48	40	0.08
geranylacetone	57	60	-0.02
β-cyclocitral	3	5	-0.2
1-nitro-3-methylbutane	59	150	-0.4
geranial	12	32	-0.4
linalool	2	6	-0.5
1-penten-3-ol	110	400	-0.6
(E)-2-pentenal	140	1500	-1.0
neral	2	30	-1.2
pentanol	120	4000	-1.5
pseudoionone	10	800	-1.9
isobutyl cyanide	13	1000	-1.9
hexanol	7	500	-1.9
epoxy-β-ionone	1	100	-2.0

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Chapter 18

Volatile Constituents of Pineapple (*Ananas Comosus* [L.] Merr.)

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The volatiles of fresh pineapple (*Ananas comosus* [L.] Merr.) crown, pulp and intact fruit were studied by capillary gas chromatography and capillary gas chromatography-mass spectrometry. The fruit was sampled using dynamic headspace sampling and vacuum steam distillation-extraction. Analyses showed that the crown contains C₆ aldehydes and alcohols while the pulp and intact fruit are characterized by a diverse assortment of esters, hydrocarbons, alcohols and carbonyl compounds. Odor unit values, calculated from odor threshold and concentration data, indicate that the following compounds are important contributors to fresh pineapple aroma: 2,5-dimethyl-4-hydroxy-3(2H)-furanone, methyl 2-methylbutanoate, ethyl 2-methylbutanoate, ethyl acetate, ethyl hexanoate, ethyl butanoate, ethyl 2-methylpropanoate, methyl hexanoate and methyl butanoate.

Pineapple flavor has been the subject of extensive studies (1). Early work has been discussed in depth in a review by Flath (2). Pickenhagen et al. (3) reported the amounts of 2,5-dimethyl-4-hydroxy-3(2H)-furanone (furanol) and its corresponding methyl ether in pineapples. With the aid of GC-sniffing, 2-propenyl hexanoate was identified in pineapple (4). Though the compound possesses a pineapple-like odor (5) it does not contribute to pineapple flavor at its naturally occurring levels in the fruit (6). In their study of the non-polar fraction Berger et al. (7) identified the sesquiterpenes α -copaene, β -ylangene, α -patchoulene (tentative), γ -gurjunene, germacrene D, α -muurolene and δ -cadinene. However, none of the compounds identified were responsible for the balsamic, fruity odor of the fraction. Nineteen new compounds including 1-(E,Z)-3,5-undecatriene and 1-(E,Z,Z)-3,5,8-undecatetraene were identified by Berger et al. (8). Due to their low odor thresholds the two unsaturated hydrocarbons may be important contributors to pineapple flavor. Ohta et al. (9) studied canned Philippine pineapple juice and reported methyl 4-acetoxyhexanoate and various carboxylic acids. The enantiomeric composition of various lactones, hydroxy and acetoxy esters occurring in pineapples has been reported by Tressl and co-workers (10,11).

Though pineapple flavor has been extensively studied relatively little work has been done on the odor properties and significance of the various constituents (6,8). This study investigates the odor contribution of various constituents. The volatiles from three parts of fresh pineapple, the crown, pulp and intact fruit were examined.

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EXPERIMENTAL SECTION

Materials. Fresh pineapples (*Ananas comosus* (L.) Merr.) var. Smooth Cayenne were obtained via air freight from Hawaii.

Dynamic Headspace Sampling. The crown, intact fruit and blended pulp volatiles were isolated using dynamic headspace sampling.

Four crowns (total weight, 765g) were placed in a 9L Pyrex glass container (a modified desiccator). A Pyrex head to allow the passage of air into and out was fitted into a standard ground glass joint in the upper part of the container. Purified air (passed through activated carbon) was passed over the leaves at a flow rate of 3L/min. The air exiting the desiccator was passed through a Tenax trap. The traps were constructed of Pyrex glass tubing and terminated in standard ball and socket joints. The traps were loaded with 10g of 60/80 mesh Tenax GC (Alltech Associates, Deerfield, IL) producing a column 14 cm long X 2.2 cm i.d. The trapping was continued for 24 h at room temperature (ca. 24 °C). The collected volatiles were eluted from the Tenax trap with 80 mL of freshly distilled diethyl ether containing ca. 0.001% Ethyl antioxidant 330 (1,3,5-trimethyl-2,4,6-tris [3,5-di-tert-butyl-4-hydroxybenzyl]benzene). The ether extract was carefully concentrated with a Vigreux column to a final volume of ca. 100 μ l.

Two intact pineapples (without crowns, total weight, 3.2 kg) were placed into a 9L glass container. The volatiles were collected, eluted and concentrated in the same manner as described above.

The pineapple pulp was sampled in the following manner. The skin was removed from the fruit. The flesh (400g) was cut into chunks and placed in a Waring blender with 200 mL of saturated CaCl_2 solution. The mixture was blended for 30 s. Fifteen milliliters of a water solution containing 20 ppm 3-heptanone and fifteen milliliters of a water solution containing 20 ppm 6-methyl-5-hepten-2-one was then added and the mixture was blended for 15 s. The mixture was placed in a 2L round-bottomed flask. Two hundred mL of water was added to the flask. Purified air (3L/min) was passed over the surface of the vigorously stirred mixture via a Teflon tube and exited out of the flask through a Tenax trap of the same dimensions as described above. The sampling apparatus is shown in Figure 1. The mixture was sampled for 3 h. The trapped material was eluted and the extract was concentrated as described above.

Vacuum Steam Distillation-Extraction. Pineapple pulp (1.0 kg) was blended with 1 L water for 20 s in a Waring blender. Three batches were prepared using a total of 3.13 kg fruit pulp. The blended material was added to a 12 L round-bottomed flask. Sixty milliliters of antifoam solution was added. The antifoam solution was prepared by adding 12 mL of Hartwick antifoam 50 emulsion to 900 mL of water in a 1 L flask and boiling until the volume was reduced to 600 mL. A modified Likens-Nickerson steam distillation extraction head (12) was employed. A 250 mL round-bottomed flask containing 125 mL hexane (containing 0.001% of Ethyl antioxidant 330) was attached to the solvent arm of the extraction head. Simultaneous steam distillation-extraction (SDE) under 60mm Hg was continued for 3 h. The resulting hexane extract was chilled to -20 °C to freeze out residual water. The extract was quickly decanted and then concentrated under 60mm Hg with a Vigreux column to a final volume of ca. 500 μ l.

Gas Chromatography. A Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Avondale, PA) with a flame ionization detector (FID), equipped with a 60 m X 0.32 mm i.d. DB-WAX column (d_f = 0.25 μ m, bonded polyethylene glycol, J&W Scientific, Folsom, CA) was employed. Helium carrier gas was used at a flow rate of 1.64 mL/min (30°C). The oven temperature was programmed from 30°C (4 min isothermal) to 180°C at 2°C/min. A split ratio of 1:28 was used. The injector and detector were maintained at 200°C and 220°C, respectively. A 60 m X 0.32 mm i.d. DB-1 column (d_f = 0.25 μ m, bonded dimethyl polysiloxane, J&W Scientific, Folsom, CA) was used to analyze the sample prepared by vacuum steam distillation-extraction. Helium carrier gas was used at a flow rate of 1.60 mL/min (30 °C). The oven temperature was programmed from 30 °C (4 min isothermal) to

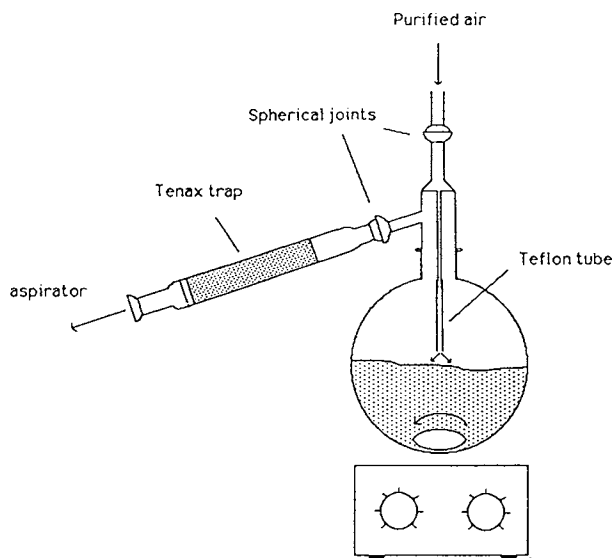


Figure 1. Apparatus for dynamic headspace sampling.

210 °C at 2 °C/min. A split ratio of 1:22 was used. Data processing was performed with an HP 5895 GC ChemStation.

Gas Chromatography-Mass Spectrometry. A Finnigan MAT 4500 GC/MS/INCOS system (Finnigan MAT, San Jose, CA) was used for analysis. A 60 m X 0.32 mm i.d. DB-WAX column was employed for the headspace samples. The oven temperature was programmed from 50°C to 180°C at 2°C/min. A 60 m X 0.32 mm i.d. DB-1 column was used for the sample prepared by vacuum SDE. The oven temperature was programmed from 30 °C (4 min isothermal) to 210 °C at 2 °C/min. Helium was used as the carrier gas at a flow rate of 3.2 mL/min. The column outlet was inserted directly into the ion source block. The instrument was operated in the electron impact mode at an ionization voltage of 70 eV. The mass spectrometer was repetitively scanned from 33 to 350 m/z in a one-second cycle.

Reference Compounds. Authentic reference compounds were obtained from commercial sources, synthesized or isolated from essential oils. Methyl 3-acetoxybutanoate was prepared by acetylation of methyl 3-hydroxybutanoate with acetic anhydride. Methyl 3-acetoxybutanoate had the following mass spectrum, m/z (relative intensity): 117(27), 100(23), 87(4), 85(11), 74(2), 69(37), 59(20), 43(100). Methyl (Z)-3-hexenoate was prepared from methyl (E)-3-hexenoate by reaction with the equilibration catalyst, benzenethiol (13). 3-Methylthiopropyl acetate was formed by acetylation of 3-methylthiopropyl with acetic anhydride. 3-Methylthiopropyl acetate had the following mass spectrum, m/z (relative intensity): 148 (43), 105(5), 101(3), 90(5), 89(5), 88(100), 75(19), 73(70), 61(44), 47(12), 45(11), 43(92). Methyl 4-acetoxyhexanoate was prepared by treatment of γ -hexalactone with base followed by acetylation with acetic anhydride. Methyl 4-acetoxyhexanoate had the following mass spectrum, m/z (relative intensity): 159(5), 145(8), 128(8), 117(51), 115(40), 113(21), 101(5), 97(7), 88(10), 85(28), 74(8), 69(11), 55(8), 43(100). Methyl 4-acetoxyoctanoate was prepared by treatment of γ -octalactone with base followed by acetylation with acetic anhydride. Methyl 4-acetoxyoctanoate had the following mass spectrum, m/z (relative intensity): 173(13), 159(14), 143(29), 141(40), 129(11), 124(13), 117(100), 115(18), 101(15), 88(16), 85(36), 74(13), 55(17), 43(99).

Odor Thresholds. These were determined on GLC purified samples using methods previously described (14), with a panel of 16-20 judges.

RESULTS AND DISCUSSION

The samples were analyzed by capillary gas chromatography and gas chromatography/mass spectrometry (GC/MS). Identifications were verified by comparing the component's mass spectrum and experimental retention index (I) with that of an authentic reference standard. The retention system proposed by Kovats (15) was utilized. When standards were not available the identifications were considered tentative.

The volatiles of the crown, pulp and whole intact fruit were examined by dynamic headspace sampling using a procedure developed in our laboratory (16). The method uses a fast flow of sweeping gas (3L/min) onto large Tenax traps.

Table I lists the compounds identified in a headspace sample of pineapple crowns. The sample was characterized by low levels of very few volatiles. The C₆ compounds, hexanal, (Z)-3-hexenal, (E)-2-hexenal and (Z)-3-hexenol were probably produced enzymatically in response to tissue damage from cutting (17). Hydrocarbons identified include styrene, and the monoterpenes, α - and β -pinene.

A GC/FID chromatogram of pulp headspace volatiles is shown in Figure 2. Components identified in the pulp along with their concentrations are listed in Table II. Quantitation was based on two internal standards, 3-heptanone and 6-methyl-5-hepten-2-one. These compounds were chosen for their chemical stability and their elution in relatively uncrowded regions of the chromatogram. Though earlier studies (16) showed good solute recovery using this sampling technique the reported concentrations should be considered as approximate values since solute recoveries and flame ionization detector

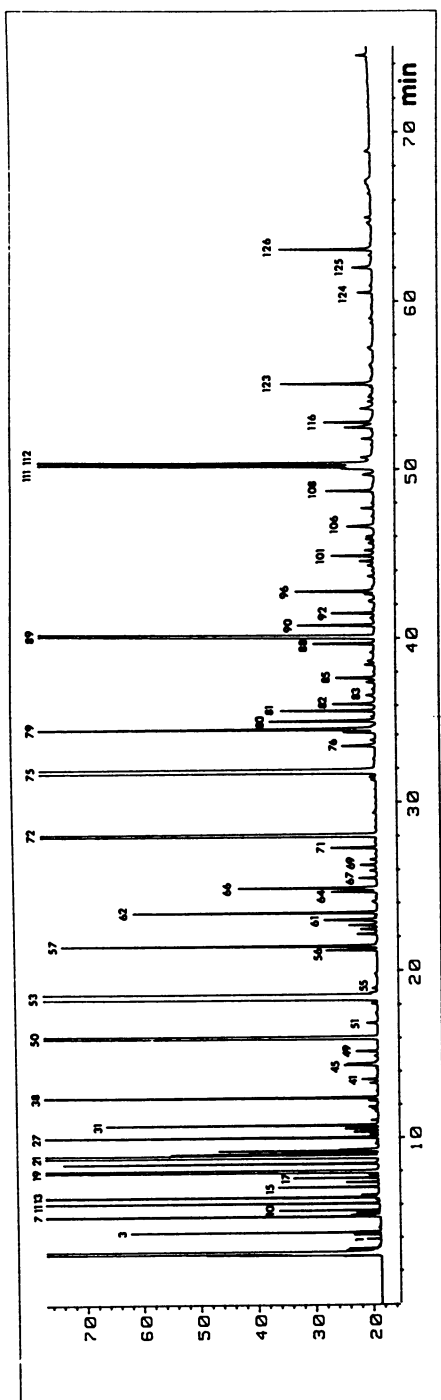


Figure 2. Capillary gas chromatogram of blended pineapple pulp volatiles obtained by dynamic headspace sampling. Temperature programmed from 30°C (4 min isothermal) to 180°C at 2°C/min on a 60m X 0.32 mm i.d. DB-WAX column. The peak numbers correspond to the numbers in Table II.

(FID) response factors were not determined for each identified pineapple constituent. Qualitative and quantitative differences were noted between samples and it was observed (by GC/MS) that ethyl heptanoate eluted as a shoulder on the 6-methyl-5-hepten-2-one peak in some runs. Therefore, the use of 6-methyl-5-hepten-2-one was discontinued in later runs. The major compounds found were methyl hexanoate, methyl 2-methylbutanoate, methyl butanoate, methyl octanoate, methyl 3-methylthiopropanoate and ethyl acetate.

Table I. Pineapple Crown Volatiles

constituent	DBWAX	
	exp.	ref.
ethyl acetate	889	890
ethanol	940	927
pentanal		975
α -pinene	1015	1012
hexanal	1081	1078
β -pinene	1096	1093
(Z)-3-hexenal	1145	1138
(E)-2-hexenal	1213	1215
styrene	1253	1252
(Z)-3-hexenal	1332	1378

To assess the relative contribution of the identified constituents to the total odor the number of odor units (U_0) was calculated. Guadagni et al. (14) defined the odor unit as the ratio of the concentration of the compound and its odor threshold. Table III lists the odor units of some pineapple constituents calculated from their concentrations and odor thresholds. Compounds are listed in decreasing order of their number of odor units. The list is dominated by esters. It is likely that ethyl acetate makes a larger contribution to pineapple flavor than is shown on the table. Preliminary experiments have indicated that the % recovery of ethyl acetate with this headspace sampling procedure is very low (approximately 5%), presumably due to breakthrough on the Tenax trap. Methyl 3-acetoxyhexanoate, methyl (E)-3-octenoate and ethanol are present in concentrations less than their odor thresholds and hence are expected to make little or no contribution to the pineapple odor. The odor thresholds for the unsaturated hydrocarbons, 1,3,5-undecatriene and 1,3,5,8-undecatetraene have not been determined and therefore they are not included on the table though they probably make an important contribution to pineapple flavor. The potent odor character of 1-(E,Z)-3,5-undecatriene and 1-(E,Z,Z)-3,5,8-undecatetraene has been described by Berger et al. (8). The configuration of the double bond in the 5 position is crucial; the corresponding isomers, 1-(E,E)-3,5-undecatriene and 1-(E,E,Z)-3,5,8-undecatetraene have odor thresholds 10^6 and 10^4 times higher, respectively (8). Another important volatile not included in Table III is 2,5-dimethyl-4-hydroxy-3(2H)-furanone (furanol). This highly polar constituent does not steam distill and was not recovered using our sampling techniques. Its contribution to pineapple flavor was estimated using values published in the literature. Pickenhagen et al. (3) found 7.4 ppm of furaneol in pineapples from the Ivory Coast. This potent odorant has an odor threshold of 0.03 ppb (18). Thus the odor unit value calculated for furaneol is approximately 2.5×10^5 . Therefore, it must be one of the major contributors to pineapple flavor. It is interesting to examine the odor descriptions of the compounds with the highest odor units (Table IV). These esters largely possess pineapple or apple odors. Ethyl 2-methylbutanoate has been found to be an important character impact compound in Delicious apple essence (19).

Constituents identified in the intact ripe pineapple headspace sample are listed in Table V. Figure 3 shows a GC/FID chromatogram of pineapple headspace. The ester fraction comprises about 81% of the total area. Quantitatively, the major constituents were methyl

Table II. Volatile Constituents of Pineapple - Blended Pulp (Headspace)

peak no. ^a	constituent	DBWAX		
		exp.	ref.	conc. ppb
7	ethyl acetate	887	886	503
10	methyl propanoate	904	904	14
11	methyl 2-methylpropanoate	922	920	64
13	ethanol	936	927	192
15	ethyl propanoate	957	952	17
16	ethyl 2-methylpropanoate	966	962	6
17	propyl acetate	974	974	17
19	methyl butanoate	984	982	2026
21	methyl 2-methylbutanoate	1007	1009	2079
22	2-methylpropyl acetate	1011	1010	41
23	chloroform	1016	1015	35
24	methyl 3-methylbutanoate	1017	1016	9
27	ethyl butanoate	1035	1035	92
31	ethyl 2-methylbutanoate	1051	1048	66
38	methyl pentanoate	1081	1083	150
46	3-methylbutyl acetate	1118	1120	8
49	ethyl pentanoate	1131	1132	6
50	3-heptanone (internal standard)			
53	methyl hexanoate	1182	1180	3442
56	(methyl 5-hexenoate) ^{b,c}	1224		15
57	ethyl hexanoate	1228	1228	99
61	(methyl (Z)-3-hexenoate) ^{b,c}	1252		22
62	methyl (E)-3-hexenoate	1258	1259	73
66	methyl heptanoate	1279	1279	48
72	6-methyl-5-hepten-2-one (internal standard)			
75	methyl octanoate	1380	1380	1451
78	methyl 2,4-hexadienoate ^c	1414	1447	11
79	(methyl (Z)-4-octenoate) ^b	1417		133
80	ethyl octanoate	1426	1426	37
81	1,3,5,8-undecatetraene+ acetic acid	1431		31
82	methyl (E)-3-octenoate	1446	1446	15
85	α -copaene	1469	1471	15
88	dimethyl malonate	1499	1500	19
89	methyl 3-methylthiopropoate	1506	1505	596
92	methyl 3-acetoxybutanoate	1529	1530	14
96	ethyl 3-methylthiopropoate	1551	1551	27
106	methyl (Z)-4-decenoate	1612	1623	14
112	methyl 3-acetoxyhexanoate	1676	1678	166
115	ethyl 3-acetoxyhexanoate	1712	1712	13
123	(methyl 5-acetoxyhexanoate) ^b	1759		33
126	methyl 5-acetoxyoctanoate	1904	1904	34

^athe peak numbers correspond to the numbers in Figure 2. ^btentative identifications enclosed in parentheses. ^cidentified for the first time in pineapple.

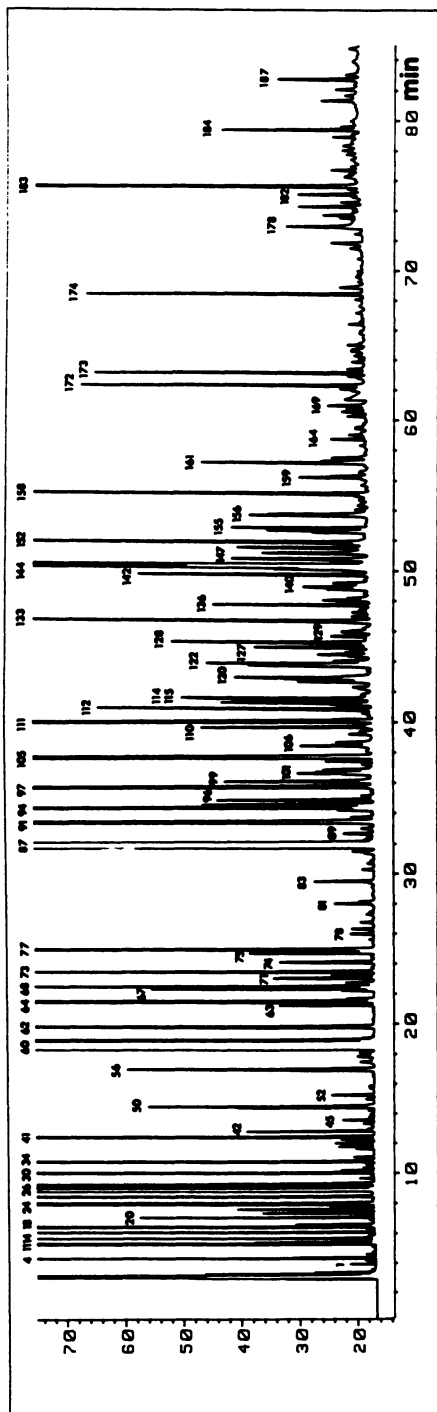


Figure 3. Capillary gas chromatogram of intact pineapple headspace volatiles. Temperature programmed from 30°C (4 min isothermal) to 180°C at 2°C/min on a 60m X 0.32 mm i.d. DB-WAX column. The peak numbers correspond to the numbers in Table V.

hexanoate, methyl 2-methylbutanoate, methyl octanoate, ethyl acetate and methyl butanoate. These esters which make up the bulk of the intact fruit headspace sample (>69%) were also major compounds found in the pulp sample. The presence of a variety of unsaturated hydrocarbons is noteworthy. This fraction accounts for about 8.6% of the

Table III. Odor Thresholds and Odor Units for Selected Constituents in Blended Hawaiian Pineapples (Pulp)

Constituent	Odor Threshold ppb	Odor Units (U ₀) ^a
methyl 2-methylbutanoate	0.25	8316
ethyl 2-methylbutanoate	0.3	220
ethyl acetate	5	100.6
ethyl hexanoate	1	99
ethyl butanoate	1	92
ethyl 2-methylpropanoate	0.1	60
methyl hexanoate	70	49
methyl butanoate	60	33.8
methyl heptanoate	4	12
methyl 2-methylpropanoate	7	9.1
methyl pentanoate	20	7.5
methyl octanoate	200	7.3
methyl (Z)-4-decenoate	3	4.7
ethyl pentanoate	1.5	4
3-methylbutyl acetate	2	4
ethyl 3-methylthiopropoate	7	3.9
methyl 3-methylthiopropoate	180	3.3
ethyl propanoate	10	1.7
methyl 3-acetoxyhexanoate	190	0.9
methyl (E)-3-octenoate	150	0.1
ethanol	100000	0.002

^aU₀ = concentration of the compound divided by its threshold concentration.

Table IV. Odor Descriptions and Odor Units for Selected Constituents in Blended Hawaiian Pineapples (Pulp)

Constituent	Odor Units	Odor Description (Fenaroli (5))
methyl 2-methylbutanoate	8316	pungent, fruity
ethyl 2-methylbutanoate	220	apple (19)
ethyl acetate	100.6	ether-like reminiscent of pineapple
ethyl hexanoate	99	powerful fruity with pineapple-banana note
ethyl butanoate	92	fruity with pineapple undernote
ethyl 2-methylpropanoate	60	apple-like
methyl hexanoate	49	ether-like reminiscent of pineapple
methyl butanoate	33.8	apple-like
methyl heptanoate	12	strong, almost fruity, orris-like

Table V. Volatile Constituents of Pineapple - Whole Intact Fruit (Headspace)

peak no. ^a	constituent	DBWAX		%area ^b
		exp.	ref.	
11	ethyl acetate	887	886	5.91
14	methyl propanoate	904	904	0.14
16	methyl 2-methylpropanoate	922	920	0.21
18	ethanol	936	927	1.56
20	ethyl propanoate	957	952	0.12
21	ethyl 2-methylpropanoate	966	962	0.06
22	propyl acetate	973	974	0.08
24	methyl butanoate	984	982	5.29
26	methyl 2-methylbutanoate	1008	1009	11.42
27	2-methylpropyl acetate	1011	1010	0.42
28	chloroform	1016	1015	0.32
29	methyl 3-methylbutanoate	1017	1016	0.09
30	ethyl butanoate	1035	1035	0.70
34	ethyl 2-methylbutanoate	1051	1048	0.63
36	ethyl 3-methylbutanoate	1067	1065	tr
37	butyl acetate ^d	1070	1068	0.03
40	hexanal	1078	1078	0.02
41	methyl pentanoate	1082	1083	0.82
42	2-methylpropanol	1088	1084	0.09
45	diethyl carbonate?	1099	1102	0.04
50	3-methylbutyl acetate	1118	1118	0.19
52	ethyl pentanoate	1131	1132	0.04
56	myrcene ^d	1160	1160	0.30
59	2-heptanone ^d	1176	1177	tr
60	methyl hexanoate	1187	1180	36.57
61	limonene ^d	1188	1183	0.22
62	3-methylbutanol	1200	1201	0.55
63	(methyl 5-hexenoate) ^{c,d}	1224		0.08
64	ethyl hexanoate	1228	1228	1.14
66	(monterpene) ^c	1239		0.03
67	(E)- β -ocimene ^d	1241	1243	0.20
68	styrene ^d	1243	1242	0.34
71	(methyl (Z)-3-hexenoate) ^{c,d}	1252		0.12
73	methyl (E)-3-hexenoate	1258	1259	0.31
74	acetoin	1267	1268	0.12
77	methyl heptanoate	1279	1279	0.76
81	(methyl 4-heptenoate) ^{c,d}	1323		0.04
83	hexanol ^d	1346	1348	0.05
87	methyl octanoate	1383	1380	10.27
88	1-(E,Z)-3,5-undecatriene	1384	1382	0.39
89	1-(E,E)-3,5-undecatriene	1391	1393	0.03
91	(sesquiterpene) ^c	1402		1.53
92	(sesquiterpene) ^c	1407		0.03
94	(methyl (Z)-4-octenoate) ^c	1418		0.89
95	(sesquiterpene) ^c	1421		0.17
96	ethyl octanoate	1426	1426	0.15
97	1,3,5,8-undecatetraene	1440		1.42
99	methyl (E)-3-octenoate	1446	1446	0.16
101	1,3,5,8-undecatetraene	1454		0.10
102	(sesquiterpene) ^c	1455		0.05

Table V. *Continued*

peak no. ^a	constituent	DBWAX		%area ^b
		exp.	ref.	
104	methyl 3-hydroxybutanoate	1466	1467	0.05
105	α -copaene	1471	1471	1.60
106	methyl nonanoate	1481	1481	0.08
108	methyl (E)-2-octenoate ^d	1484	1485	0.05
110	dimethyl malonate	1499	1500	0.18
111	methyl 3-methylthiopropoate	1506	1505	1.47
114	methyl 3-acetoxybutanoate	1529	1530	0.27
115	2,3-butanediol ^d	1529	1536	e
119	ethyl 3-methylthiopropoate	1550	1551	0.08
120	(sesquiterpene) ^c	1552		0.19
122	β -copaene ^d	1567	1567	0.17
127	methyl decanoate	1583	1584	0.11
128	(sesquiterpene) ^c	1589		0.22
133	methyl (Z)-4-decenoate	1612	1623	0.42
136	methyl 3-hydroxyhexanoate	1630	1632	0.16
142	γ -muurolene ^d	1664	1665	0.27
145	methyl 3-acetoxyhexanoate	1676	1678	0.66
147	(sesquiterpene) ^c	1682		0.20
148	β -selinene ^d	1688	1689	0.16
150	α -selinene ^d	1694	1694	0.16
152	α -muurolene	1700	1700	0.46
153	ethyl 3-acetoxyhexanoate	1712	1712	0.05
154	(sesquiterpene) ^c	1714		0.12
156	δ -cadinene	1732	1734	0.15
158	(methyl 5-acetoxyhexanoate) ^c	1759		0.45
161	2-phenylethyl acetate ^d	1794	1794	0.18
172	2-phenylethanol	1889	1890	0.30
173	methyl 5-acetoxyoctanoate	1904	1904	0.27
183	(ethylphenol) ^{c,d}	2155		0.97

^athe peak numbers correspond to the numbers in Figure 3. ^bpeak area percentage of the total FID area excluding the solvent peaks (assuming all response factors of 1). "tr" represents less than 0.02%.

^ctentative identifications enclosed in parentheses.

^didentified for the first time in pineapple. ^emerged with previous peak.

Table VI. Volatile Constituents of Pineapple - Blended Pulp (Vacuum SDE)

peak no.	constituent	DB1		%area ^a
		exp.	ref.	
1	methyl butanoate	709	705	0.55
2	methyl cyclohexane ^b	719	718	0.14
4	dimethyl hexane ^b	735		0.21
5	dimethyl hexane ^b	737		0.08
7	2-methylpropyl acetate	764	764	0.04
9	methyl 2-methylbutanoate	768	768	1.03
10	diethyl carbonate	769	769	0.02
13	3-hexanol	784	784	0.01
14	ethyl butanoate	788	789	0.43
16	methyl pentanoate	810	810	0.24
18	ethyl 2-methylbutanoate	842	842	0.29
19	ethyl 3-methylbutanoate	844	845	0.01
21	3-methylbutyl acetate	866	866	0.17
22	2-methylbutyl acetate	868	869	0.08
23	ethyl pentanoate	888	888	0.10
24	(methyl 5-hexenoate) ^{c,d}	894		0.06
25	dimethyl malonate	899	897	0.06
27	3-methylbut-2-enyl acetate ^d		902	tr
28	methyl hexanoate	914	910	14.72
29	(methyl (Z)-3-hexenoate) ^{c,d}	917	916	0.05
30	ester	918		0.04
31	methyl (E)-3-hexenoate	921	920	0.51
33	methyl (E)-2-hexenoate	947	948	0.10
37	ethyl hexanoate	988	986	6.80
39	methyl 2,4-hexadienoate ^d	991	991	0.06
40	methyl 3-methylthiopropanoate	1000	992	17.41
41	γ -hexalactone	1003	1003	0.06
42	methyl heptanoate	1010	1009	0.20
43	methyl 3-acetoxybutanoate	1017	1016	0.35
45	methyl 3-hydroxyhexanoate	1029	1026	0.03
46	(Z)- β -ocimene ^d	1030	1026	0.02
47	2,5-dimethyl-4-methoxy-3(2H)-furanone	1032	1031	0.05
48	(E)- β -ocimene ^d	1041	1037	0.13
52	ethyl 3-methylthiopropanoate + ?	1075	1072	3.19
55	ethyl heptanoate	1084	1080	0.10
56	linalool?	1086	1083	0.04
58	3-methylthiopropyl acetate ^d	1091	1091	0.10
60	methyl (Z)-4-octenoate	1097	1097	0.77
62	ester	1101		0.03
63	ethyl 3-hydroxyhexanoate	1105	1103	0.05
64	methyl octanoate + methyl (E)-3-octenoate	1111	1107	6.82
68	methyl phenylacetate ^d	1145	1144	0.10
69	methyl (E)-2-octenoate ^d	1150	1150	0.03
71	4-terpineol	1158	1159	0.07
72	1-(E,Z)-3,5-undecatriene+ 1-(E,Z,Z)-3,5,8-undecatetraene	1165	1165	0.70
73	α -terpineol	1169	1170	0.07
74	ethyl (Z)-4-octenoate?	1173		0.38
75	1-(E,E,Z)-3,5,8-undecatetraene	1175	1175	0.06
78	methyl 3-acetoxyhexanoate + ?	1186	1176	15.17

Table VI. Continued

peak no.	constituent	DB ¹		%area ^a
		exp.	ref.	
79	methyl 4-acetoxyhexanoate	1205	1203	0.56
80	methyl nonanoate	1207	1207	0.13
82	(methyl 5-acetoxyhexanoate) ^c	1226		1.33
85	ethyl 3-acetoxyhexanoate	1252	1250	2.24
87	ethyl 4-acetoxyhexanoate?	1275		0.19
88	ethyl nonanoate	1282	1279	0.04
89	methyl (Z)-4-decenoate + ?	1291	1289	3.09
90	ethyl 5-acetoxyhexanoate	1293	1293	0.16
92	methyl decanoate	1308	1307	0.38
94	(sesquiterpene) ^c	1321		0.14
95	(sesquiterpene) ^c	1327		0.11
98	cyclocopacamphene ^d	1360	1361	0.13
99	(sesquiterpene) ^c + ?	1363		1.44
100	(methyl 3-acetoxyoctanoate) ^c	1365		0.28
101	α -copaene	1370	1370	1.72
103	methyl 4-acetoxyoctanoate + ethyl decanoate	1381	1379	0.80
104	methyl 5-acetoxyoctanoate	1389	1387	2.36
105	(α -gurjunene) ^{c,d}	1399	1401	0.19
107	β -copaene ^d	1418	1418	0.16
108	(sesquiterpene) ^c	1422		0.22
111	ethyl 3-acetoxyoctanoate?	1433		0.09
114	ethyl 4-acetoxyoctanoate?	1447		0.09
115	ethyl 5-acetoxyoctanoate	1457	1458	0.70
116	γ -muurolene ^d + (sesquiterpene) ^c	1465	1464	0.75
121	α -selinene ^d + (sesquiterpene) ^c	1482	1489	0.38
122	α -muurolene	1488	1487	1.51
123	(sesquiterpene) ^c	1489		0.64
127	(sesquiterpene) ^c	1501		0.08
128	δ -cadinene	1508	1513	0.51
131	calacorene?	1520		0.05
137	ethyl dodecanoate	1579	1578	0.06
146	γ -dodecalactone	1631	1635	0.04
151	geranyl hexanoate?	1731		0.04
156	ethyl tetradecanoate	1778	1778	0.02
160	ethyl hexadecanoate	1978	1978	0.02

^apeak area percentage of the total FID area excluding the solvent peaks (assuming all response factors of 1). the values are approximate since there are known pineapple constituents co-eluting with the solvent peaks. "tr" represents less than 0.01%. ^bsolvent contaminant. ^ctentative identifications enclosed in parentheses. ^didentified for the first time in pineapple.

total area. This sampling procedure is well suited for the analysis of unsaturated hydrocarbons which may undergo enzymatic and/or oxidative degradation with conventional sampling and separation techniques (20). Monoterpene hydrocarbons were identified for the first time in pineapple. These include myrcene, limonene and (E)- β -ocimene. None of these compounds were found in the pulp sample. Similarly, Takeoka et al. (21) identified a number of monoterpene hydrocarbons in intact nectarine headspace while none were found in a sample prepared by vacuum distillation followed by liquid-liquid extraction.

Previous studies (Z) showed evidence for the presence of at least 20 sesquiterpene hydrocarbons in pineapple. These researchers identified 7 sesquiterpenes (1 tentative). We confirmed the presence of 3 of the sesquiterpenes and identified β -copaene, γ -muurolene, α - and β -selinene in addition. Mass spectral data indicated the presence of other sesquiterpene hydrocarbons and oxygenated sesquiterpenes which we have been unable to characterize at present.

Methyl (E)-2-octenoate was found for the first time in pineapple. It had been previously reported in pears (22) and soursop (23). Its formation from methyl (E)-3-octenoate (a pineapple constituent) by 2,3-(E,E)-enoyl-CoA-isomerase was postulated by Berger and Kollmannsberger (6).

The vacuum SDE method confirmed the presence of nearly all of the constituents identified using dynamic headspace sampling and revealed many additional compounds. The method was more effective in extracting the less volatile constituents such as long chain esters. In contrast to the previous runs this sample was chromatographed on a non-polar DB-1 column. The constituents identified in the pulp sample prepared by vacuum SDE are listed in Table VI. The %area values should be considered as only approximate since known pineapple constituents such as ethyl acetate, methyl propanoate, methyl 2-methylpropanoate, ethyl propanoate, ethanol, propyl acetate, and ethyl 2-methylpropanoate co-elute with the solvent peaks and hence could not be included in the quantitation.

The sulfur containing ester, 3-methylthiopropyl acetate, is reported for the first time in pineapple. This compound bears a relationship to the major esters, methyl and ethyl 3-methylthiopropanoate. It has been previously identified in apples (24), beer (25), wine (26) and whisky (27). The sesquiterpene hydrocarbon, cyclocopacamphene, is another newly reported pineapple constituent. This constituent was previously reported in vetiver oil (28).

The presence of the recently reported ester, methyl 4-acetoxyoctanoate (29) was confirmed.

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