Carotenoid-Derived Aroma Compounds

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Carotenoid-Derived Aroma Compounds

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Foreword

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Preface

The field of carotenoid research has expanded enormously over the years, spreading into many diverse areas of science. Although many aspects of carotenoid research (e.g. analysis, function, biosynthesis, occurrence, and nutrition) are comprehensively covered in recent monographs, one aspect is still missing—the aroma chemistry of carotenoid-derived compounds. Up to this time, the literature on this topic has been scattered and many scientists have been unaware and unable to appreciate the crucial role carotenoids have in flavor formation. In this book, we have attempted to fill this gap by highlighting the importance that carotenoid metabolites have in the flavor and fragrance industry.

After an overview on the topic, analytical and sensory characteristics of carotenoid-derived aroma compounds are presented in Chapters 2–5. The next four chapters focus on biogenesis, means of biotechnological production as well as thermal formation. Comprehensive information about the occurrence and generation of carotenoid-derived aroma compounds in tobacco, tea, flower scents, fruits, and spices as well as grapes and wine are presented in Chapters 10–21.

The symposium on which this volume is based was sponsored by the American Chemical Society (ACS) Division of Agricultural and Food Chemistry and was presented at the 219th National Meeting of the ACS from March 26–30, 2000, in San Francisco, California. At the time of the symposium, little information was available concerning carotenoid cleavage enzymes. It has only been in the past 12 months that the understanding of carotenoid metabolism has seen a breakthrough by reports characterizing the enzymes that are responsible for the well-known and nutritionally significant cleavage of β -carotene into retinal (*J. Biol. Chem.*, **2000**, 275, 11915–11920 and *Biochem. Biophys. Res. Comm.* **2000**, 271, 334–336). This clearly demonstrates the dynamic activity in this challenging research field.

We especially thank our chapter authors and reviewers who have contributed to the success of the symposium upon which this book is based. Finally, we gratefully acknowledge the financial support from the following sources: E & J Gallo Winery, Givaudan-Roure, and Haarmann & Reimer GmbH as well as the ACS Division of Agricultural and Food Chemistry. It would not have been possible to pull together the worldwide authorities in this area were it not for their support.

The Nobel laureate, Paul Karrer, wrote in his memoirs: "The carotenoids are the most interesting class of compounds with which I have worked, and they have given me a great deal of pleasure." Carotenoids have been studied primarily because of their value as pigments, antioxidants, or nutrients. That carotenoids can also function as aroma precursors adds a surprising new dimension to carotenoid research. We hope that our readers will share our fascination and pleasure in understanding this complex family of aroma precursors.

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Chapter 1

Carotenoid-Derived Aroma Compounds: An Introduction

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> This chapter gives an introduction to the field of carotenoidderived aroma compounds and illustrates the importance of short-chain carotenoid metabolites as flavor and fragrance substances. Although carotenoid-derived aroma compounds (so-called *norterpenoids* or *norisoprenoids*) are ubiquitous constituents in plant derived aromas, very little is known about their biogeneration. Hence, possibilities for an efficient biotechnological production are still limited. After a brief summary of the historical development of norisoprenoid chemistry, this introductory chapter reviews the present knowledge about the occurrence and formation of carotenoidderived aroma compounds in natural tissues and discusses possibilities for a biotechnological production.

A considerable number of apparently carotenoid-derived compounds has been identified in nature, many of them playing an important role as flavor and fragrance substances (1). Striking examples are the unusually potent norisoprenoid fragrances β -ionone 1 and β -damascenone 2 with flavor thresholds of 0.007 ppb and 0.002 ppb, respectively. Structurally both compounds possess a

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megastigmane carbon skeleton. Differences are due to the position of the oxygen function which is located at carbon atom 9 in the ionone family and at carbon atom 7 in the damascone family (cf. Figure 1). In addition, norisoprenoids lacking an oxygen function in the side chain are traditionally referred to as megastigmanes. One example is the passion fruit constituent megastigma-4,6,8-triene **3**, which has been reported to possess a 'rose- and raspberry-like' aroma (2). Apart from ionones, damascones and megastigmanes, many other compounds with highly diverse structural features have been described, including spiroethers, acetals, and benzofurans, such as, e.g., the wine and tobacco constituents vitispirane **4** and oxoedulan **5**, respectively. In addition to the most widespread thirteen carbon (C_{13}) norisoprenoids, volatile carotenoid metabolites with a C₉, C₁₀, or C₁₁ carbon skeleton are also frequently detected in nature. Examples are shown in Figure 1.

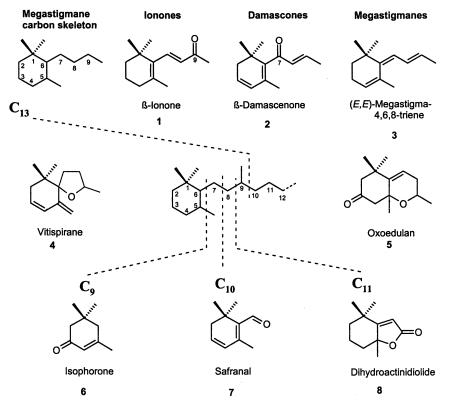


Figure 1. Different classes of degraded carotenoids and examples for potent odorants from the classes of C_9 -, C_{10} -, C_{11} -, and C_{13} -norisoprenoids.

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 β -Ionone 1 was synthesized as early as 1893 by Tiemann and Krüger (3) in an attempt to clarify the structure of the key flavor compounds of orris (Iris *pallida*) oil - the so-called irones. But it was not before 1929 that β -ionone 1 was isolated from a natural source, i.e. Boronia megastigma (4). Five years later, picrocrocin, the B-D-glucoside of hydroxysafranal, was identified as precursor of safranal 7 in stigmas of Crocus sativus by Kuhn and Winterstein (5). With the advent of gas chromatography and the development of GC-MS coupling, the number of identified norisoprenoid constituents dramatically increased and during the period 1960-1980 many important discoveries in the field of carotenoid-derived aroma compounds were made. A major breakthrough was the identification of rose ketones, i.e. β -damascenone 2 and β damascone, in Bulgarian rose (Rosa damascena) oil by Demole and coworkers in 1967, - the original results being published 20 years later, in 1987 (6,7). Because of their extraordinarily diversified odor profiles and their extreme potency, rose ketones have become one of the most important discoveries in the field of flavor and fragrance substances (7,8). Apart from essential oils (1), carotenoid-derived aroma compounds were identified in many additional sources, such as, e. g., tobacco, tea, fruits and wine. For reviews cf. Refs. 9-12. During the last two decades, the focus of norisoprenoid research shifted towards non-volatile constituents. The finding of reactive carotenoid cleavage products, which accumulate in plants mainly as glycosides, has stimulated many studies on these important aroma precursors. In combination with biomimetic studies, pathways of formation for many of the key norisoprenoids from such reactive progenitors could be established (13-15). Despite these efforts, many questions remained unanswered especially with regard to the initial steps of carotenoid cleavage. Up to now, there is more speculation than sound experimental knowledge concerning enzyme-mediated cleavage of the carotenoid chain (16,17).

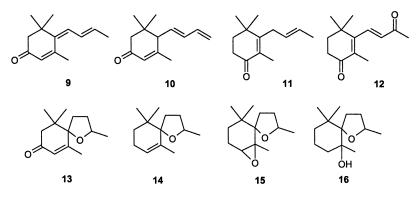
Occurrence of Norisoprenoid Aroma Compounds

Carotenoid-derived aroma compounds have not only been detected in leaf products, such as tobacco, tea, and mate, but also in many essential oils, fruits (grapes, passionfruit, starfruit, quince, apple, nectarine), vegetables (tomato, melon), spices (saffron, red pepper), as well as additional sources such as wine, rum, coffee, oak wood, honey, seaweeds etc. It is not within the scope of this presentation to provide a comprehensive review on the distribution of carotenoid-derived aroma compounds in nature, only some examples that illustrate the importance of norisoprenoids as flavor and fragrance substances as well as their widespread occurrence in nature will be presented.

Tobacco and Tea

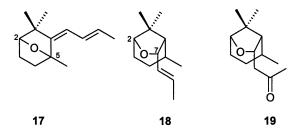
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Tobacco (*Nicotiana tabacum*) is one of the richest sources for degraded carotenoids, with almost 100 components being identified (12,13 and Refs. cited). Isomeric megastigmatrienones 9 and 10 are considered as character-impact compound of *Burley* tobacco. Other oxygenated derivatives such as, e.g., megastigma-5,8-dien-4-one 11 and 4-oxo- β -ionone 12 were also reported as important constituents of tobacco flavor (12). Among tea (*Camellia sinensis*) volatiles, isomeric ionones and damascones have been identified (18). Typical for tea aroma is the C₁₃-spiroether theaspirone 13 (19) with a sweet floral, tea-like note. The fruity smelling theaspirane 14 as well as oxygenated theaspirane derivatives 15 and 16 are further carotenoid-derived constituents that have been identified in tea flavor (20).



Essential Oils

Numerous norisoprenoids have been recognized as essential oil constituents (1,21). Known to be rich in carotenoid-derived structures are the flowers of *Osmanthus fragrance* (22) and *Boronia megastigma* (23-26). Kaiser and Lamparsky (22) identified almost one hundred constituents in an Osmanthus absolue including oxygenated theaspirane derivatives as well as a novel series of cyclic ethers **17-19** which are unique to *Osmanthus* flavor (27,28).

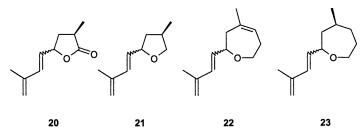


Fruits

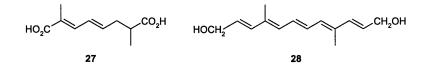
Carotenoid metabolites are common constituents in fruit flavors. In the case of quince fruit, starfruit, white-fleshed nectarines and purple passion fruit, they are considered to be particular important for the typical aroma of the fruit.

Quince fruit

The ripe fruit of quince (*Cydonia oblonga* Mill.) imparts a powerful and characteristic flavor. Among the volatile constituents a series of C_{10} constituents of irregular isoprenoid structures have been identified as key flavor substances. They have been named marmelo lactones **20** and marmelo oxides **21**, respectively (*29,30*). In addition, trace amounts of the structurally related C_{12} -ethers, the so-called quince oxepine **22** and quince oxepanes **23**, have been more recently identified in quince brandy by Näf and Velluz (*31*).



Quince volatiles 20-23 are derived from the non-volatile progenitors 24-26 that could be isolated from quince juice (cf. Figure 2). The ß-glucose ester of (4E,6E)-2,7-dimethyl-8-hydroxy-octadienoic acid 24 and the ß-glucopyranoside of diol 25 were identified as precursors of marmelo lactones 20 and marmelo oxides 21, respectively (32-34). Glycosidically bound bifunctional precursor 26 was found to give rise to a formation of quince oxepine 22 under acidic conditions (34,35). The structure of marmelo lactone precursor 24 has recently been confirmed by synthesis (36). Although no labeling experiments have been carried out so far, it is most likely that the irregular isoprenoids 24-26 are derived from the central portion of the carotenoid chain as outlined in Figure 2 (34,37). It should be noted that related carotenoid metabolites have previously been identified in tomatoes and rose petals. From tomato, the C₁₀-fragment 2,7dimethyl-octa-2,4-dienedioic acid 27 was obtained (38), whereas from rose petals the C₁₄-diol Rosafluine 28 was isolated (39).



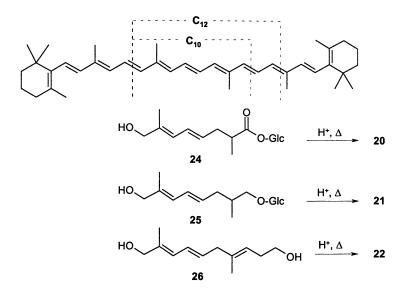
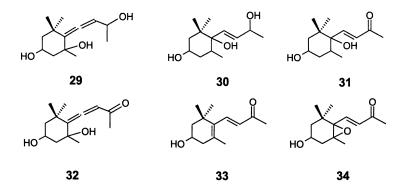


Figure 2. Postulated cleavage of quince carotenoids rationalizing the formation of C_{10} - and C_{12} -bifunctional cleavage products 24-26, which have been recognized as progenitors of quince volatiles 20-22 (for details cf. text).

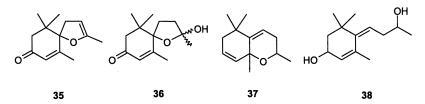
Starfruit, White-fleshed Nectarines, Purple Passionfruit

A considerable number of C_{13} -norisoprenoid volatiles has been identified in starfruit (*Averrhoa carambola* L.) including β-damascenone 2, isomeric megastigma-4,6,8-trienes 3, megastigma-4,6,8-triene-3-ones 9, and megastigma-5,8dien-4-one 11 (40,41). For the majority of starfruit norisoprenoids, secondary formation through degradation of acid-labile glycosides, involving, e.g., glycosidically bound compounds 29-34, could be established (42 and Refs. cited).



The detection of additional C_{15} -carotenoid metabolites together with C_{10} -fragments from the central part of the carotenoid chain indicated the action of two different types of carotenoid cleavage enzymes in starfruit: (i) a highly specific dioxygenase which cleaves only epoxycarotenoids along the 11,12 - double bond giving rise to C_{15} -metabolites (i.e. abscisic acid derivatives) and (ii) a 9,10-dioxygenase that accepts various carotenoids and liberates, e.g., the C_{13} -endgroups grasshopper ketone **32**, 3-hydroxy- β -ionone **33**, and 3-hydroxy-5,6-epoxy- β -ionone **34** (42).

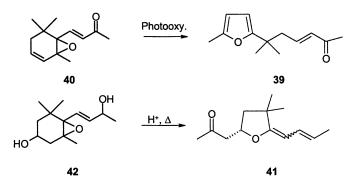
Results published by Takeoka and coworkers showed the importance of carotenoid-derived compounds for the flavor of white-fleshed nectarines (Prunus persica Batsch var. nucipersica Schneid), with b-ionone 1 and bdamascenone 2 being major aroma contributors (43). The assumption of Engel et al. (44) that 8,9-dehydrotheaspirone 35 may contribute to the typical 'flowery-note' of the white-fleshed cultivars could not be confirmed in a recent study (45). Both of the pure enantiomers of 35 were found to be too weak for odor evaluation using GC sniffing. Moreover, spiroether 35 was found to be an isolation artifact that is formed from the reactive hemiacetals 36 (45). A similar observation has also been made for isomeric edulans 37 which have been described as key flavor substances of purple passion fruit (Passiflora edulis Sims) (46,47). 3-Hydroxy-retro- α -ionol 38 was elucidated as genuine precursor of edulans 37 (48), and for an authentic sample of rac-37, Weyerstahl and Meisel (49) determined a 'weak, woody, and camphoraceous note' instead of the previously described 'sweet, floral, rose-like note'. Based on this observation, isomeric edulans 37 obviously do not play any decisive role in the flavor of passionfruit juice. It is also worth mentioning that Weyerstahl's group has published further studies on structure-odor correlations for a whole series of C_{13} -norisoprenoids as well as their homologs (50-52).



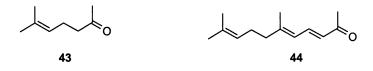
Vegetables

Only recently the furan derivative **39** has been isolated from spinach (*Spinacia oleracea* L.) leaves by Näf and coworkers (53). Compound **39** has interesting olfactory properties ('freshly cut carrots, woody orris-like note') and was previously only described as photooxidation product of C_{13} -epoxide **40** (54,55). Additional furan derivatives, i.e. isomeric 2-(2-butenylidene)-3,3-

dimethyl-5-(2-oxopropyl) tetrahydrofurans 41, have been identified in leaves of stinging nettle (*Urtica dioica* L.). E/Z-isomeric furans 41 were found to be degradation products of 3-hydroxy-5,6-epoxy- β -ionol 42 (56).



Typical for tomato (*Lycopersicon esculentum* Mill.) flavor is the presence of acyclic carotenoid cleavage products that are derived from lycopene, such as, e.g., 6-methyl-5-hepten-2-one **43** and pseudoionone **44**. From a sensory point of view, products of β -carotene and neoxanthin cleavage, i.e. β -ionone **1** and β -damascenone **2**, are far more important for tomato flavor (57).

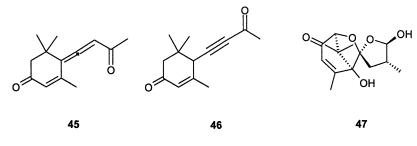


Wine

Carotenoid-derived aroma compounds were also recognized as important aroma contributors in both red and white wines and grape juices, including the Chardonnay, Chenin blanc, Semillon, Sauvignon blanc, Riesling, Cabernet Sauvignon, and Shiraz varieties (for details cf. refs. 58-65). In the case of Chardonnay juice, seventy percent of the total concentration of volatile secondary metabolites comprised C₁₃-norisoprenoids, and these were mainly observed in the acid- and the glycosidase enzyme-released fractions (59). Acid hydrolysis of the glycosidic pool of Chardonnay juice produced volatiles exhibiting some of the key varietal aromas ('tea, lime, honey') that a wine prepared from the juice also possessed. This observation clearly shows the importance of norisoprenoid aroma precursors for the flavor development in Chardonnay wines (59). Thirty-one norisoprenoid compounds have furthermore been identified by Sefton and coworkers (66) as constituents of oak woods used in wine and spirit maturation, and Masson and coworkers (67) demonstrated the presence of carotenoids in cooperage oak. Since sensory studies have not yet been reported, the contribution of oak norisoprenoids to wine flavor remains uncertain.

Honey

Extractives of New Zealand honeys have been reported to contain a diverse range of carotenoid-derived aroma substances which have been shown to be of significant interest as floral source markers. Members of the classes of C_{8} -, C_{9} -, C_{13} - and C_{15} -norisoprenoids have been identified in heather honey by Tan and coworkers, including the allenic and acetylenic diketones **45** and **46** (*68*). Broom et al. (*69*) reported the isolation and structural characterisation of the unusual C_{14} -spiroketal **47** in Kamahi (*Weinmannia racemosa*) honey. More recently, a variety of carotenoid-derived aroma compounds have been identified in various Australian honey extractives (*70*) as well as in strawberry tree (*Arbutus unedo* L.) honey (*71*).



Edible Algae

In their analysis of the algae *Porphyra tenera* ('*Asakusa-nori*'), one of the most popular edible seaweed in Japan, Flament and Ohloff (72) were able to identify a complex series of carotenoid metabolites (cf. Figure 3). As C₉-norcarotenoids 2,2,6-trimethylcyclohexanone **48**, 2,2,6-trimethylcyclohexane-1,4-dione **49**, 2-hydroxy-2,6,6-trimethylcyclohexanone **50**, 4-hydroxy-2,6,6-trimethylcyclohexanone **51**, and 4-hydroxy-2,6,6-trimethyl-2-cyclohexen-1-one **52** were detected. The group of C₁₀-norisoprenoids consisted of β-cyclocitral **53**, α-cyclocitral **54**, and safranal **7**. The C₁₁-aldehydes β-homocyclocitral **55** and 3-hydroxy-β-homocyclocitral **56** were reported for the first time in a food together with dihydroactinidiolide **8** and 2,2-dimethyl-4-(2-oxopropyl)cyclopentanone **57**. Among the group of C₁₃-norisoprenoids (compounds **1**, **58-64**, cf. Figure 3),

 β -ionone 1 and its α -isomer 58 were found to play a decisive role, explaining the delicate flowery note evolved from nori seaweed.

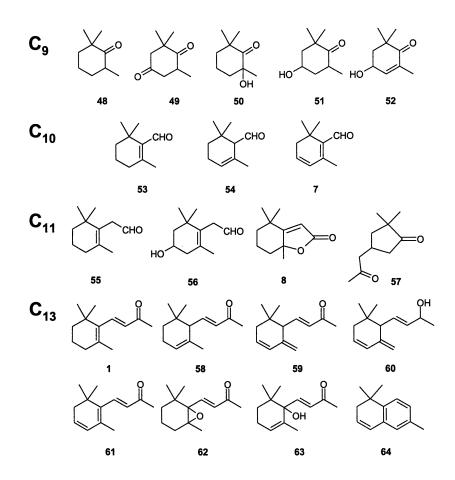


Figure 3. Carotenoid-derived compounds identified in Asakusa-nori flavor by Flament and Ohloff (72).

The Role of Carotenoids as Flavor and Fragrance Precursors

Carotenoids represent one of the most widely distributed and structurally diverse classes of natural pigments, now including some 600 structurally unique

pigments (73). Apart from crucial functions in photosynthesis, photoprotection, and nutrition, carotenoids are considered to be important aroma precursors. Aroma generation from carotenoids is thought to proceed via enzymatic and non-enzymatic pathways. Non-enzymatic cleavage includes photo-oxygenation (74,75), (auto)oxidation (75-78) as well as thermal degradation processes (79-83). The biodegradation of carotenoids is assumed to be catalyzed by dioxygenase systems (13,16,17).

Whereas detailed knowledge is available concerning the biosynthesis of carotenoids in plants (84), astonishing little is known about the metabolic pathways that lead to the formation of aroma compounds. In general three steps are required to generate an aroma compound from the parent carotenoid (i) the initial dioxygenase cleavage, (ii) subsequent enzymatic transformations of the initial cleavage product giving rise to polar intermediates (aroma precursors), and (iii) acid-catalyzed conversions of the non-volatile precursors into the aroma-active form (16). One example illustrating these reaction is the formation of β -damascenone from neoxanthin (cf. Figure 4). The primary oxidative cleavage product of neoxanthin, grasshopper ketone 32, has to be enzymatically reduced before finally being acid-catalyzed converted into the odoriferous ketone 2. In very limited cases, i.e. formation of α - and β -ionone 1, the target compound is already obtained after the initial cleavage step.

For step I and II of carotenoid cleavage (cf. Figure 4) extremely little knowledge is available. Some insight into the enantioselectivity of the enzymatic conversion of the primary cleavage products (step II) has been obtained through determination of the chiral composition of norisoprenoids in natural substrates (for details cf. Refs. 16,85). But almost no information is available concerning the postulated dioxygenase systems (step I). Only in rare cases, e.g. for the cyanobacterium *Microcystis*, has the carotenoid degrading system been partially characterized. In *Microcystis*, a membrane bound, cofactor-independent, iron-containing dioxygenase has been identified which cleaves carotenoids specifically at the C(7,8)- and C(7',8') double bonds (86). In the case of β -carotene, the C₁₀-aldehyde β -cyclocitral **53** and the C₂₀-pigment crocetindial are obtained as cleavage products in a 2:1 molar ratio. A similar enzyme is expected to be responsible for zeaxanthin cleavage in saffron (87,88).

Bio-oxidative cleavage reactions involving a 9,10 (9',10')-dioxygenase are plausible for the formation of C_{13} -norisoprenoids. In addition to the C_{13} endgroups several C_{27} -apocarotenoids are known to occur in fruit tissues (16). Based on these findings, a two-step mechanism has been proposed for the action of the 9,10 (9',10')-dioxygenase in rose flowers (89). The enzyme which is obviously not specific for any particular carotenoid endgroup is assumed to split the carotenoid chain regioselectively giving rise to C_{13} endgroups as well as C_{27} fragments. The C_{27} -fragment is assumed to be also accepted as substrate and in a second dioxygenase cleavage C_{13} - and (bifunctional) C_{14} -fragments are obtained (for details cf. 16,17,89).

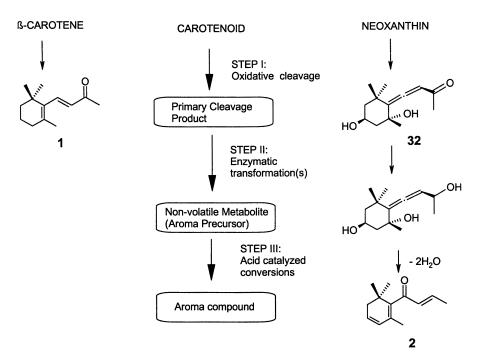


Figure 4. General steps for the conversion of carotenoids into flavor compounds, and - as examples - the formation of β -ionone 1 and β damascenone 2 from β -carotene and neoxanthin, respectively.

Biotechnological Production of Norisoprenoids

In general, three major biotechnological methods can be applied to the production of carotenoid-derived aroma compounds. These are the use of enzymes, the use of microorganisms, and the use of plant tissue or cell cultures.

As mentioned above, norisoprenoids occur predominantly in bound forms in plant tissues. This offers potential for the use of glycosidases and/or other hydrolases. Problems encountered with the enzymatic liberation of bound norisoprenoids have been discussed in Ref. 90. With regard to practical applications, one should also bear in mind that most of the commercially available enzyme preparations tolerate only low sugar and ethanol concentrations and some of them exhibit only low activity at natural pH values of fruit juices. Detailed properties of enzyme preparations used for the hydrolysis of glycosides have been published previously (90,91 and Refs. cited).

A different enzymatic approach for the biotechnological production of norisoprenoid compounds uses the carotenoid fraction of plants directly. In this case, the cleavage of the carotenoid chain is carried out by 'co-oxidation' using lipoxygenase (LOX) or other oxidase systems, such as, e.g., phenoloxidase, lactoperoxidase, and more recently xanthine oxidase (16 and Refs. cited). By far most co-oxidation studies have been carried out with lipoxygenase (linoleate: oxygen oxidoreductase, EC 1.13.11.12), a thoroughly studied enzyme that catalyzes the oxidation of polyunsaturated fatty acids containing a Z,Z-1,4pentadiene subunit to a conjugated dienoic hydroperoxide. The well-known capacity of LOX to co-oxidize carotenoids has been ascribed to the fact that a large proportion of the initially formed peroxyl radicals is not directly converted into the hydroperoxides (92). The former remain as aggressive radicals being able to attack activated sites of the polyene chain, thus leading *inter alia* to the formation of volatile break-down products. In the case of β -carotene, formation of β -ionone 1, 5,6-epoxy- β -ionone, and dihydroactinidiolide 8 as major volatile products is observed. Experimental support for the suggested free radical mechanisms has been obtained in stereochemical studies, in which no enantioselectivity for LOX catalyzed co-oxidation reactions was observed (93, 94).

Co-oxidation requires the enzyme (LOX), polyunsatured fatty acids and the polyene compounds. Since an almost equal co-oxidative reactivity of LOX isoenzymes under aerobic conditions has been reported (94), a crude mixture of soybean LOXs can be used. With regard to polyene compounds, natural sources rich in carotenoids, such as palm oil, plant extracts (e.g. carrots) or extracts from the algae *Dunaliella* are suitable starting materials. Initially, most co-oxidation reactions have been carried out in aqueous solutions by solubilizing linoleate as well as the carotenoid with different detergents (95). Today an improved co-oxidation procedure is applied to the industrial production of natural C₁₃-flavoring material (96), with maximum yields of β - and α -ionone being in the 200 mg range per kg of multiphase reaction mixture. The latter consists of carrot juice as carotenoid source, soy flour as source of LOX and vegetable oil as source of unsaturated fatty acids.

For the subsequent conversion of the co-oxidation products plant cell cultures and microorganisms can be used. Examples are presented in the literature (16,97).

Acknowledgement

PW thanks his coworkers, who are identified in the references.

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Chapter 2

Analysis of Norisoprenoid Aroma Precursors

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> Many norisoprenoid volatiles are known to be derived from reactive progenitors such as glycoconjugates and polyols. In this chapter an overview on pathways of formation of important carotenoid-derived aroma compounds from the respective non-volatile aroma precursors will be presented. Furthermore strategies for the identification and isolation of polar progenitors employing countercurrent chromatography (CCC) as well as HPLC-MS/MS are described. Applications of these analytical strategies are presented, i.e. the identification of norisoprenoid aroma precursors in saffron, Gardenia (Gardenia jasminoides) fruits and apple (Malus domestica) leaves.

Studies starting in the 1980's revealed for the first time that many volatiles including carotenoid-degradation products are not genuine plant constituents but rather are formed from less or non-volatile precursors, such as polyhydroxylated as well as glycosidically bound compounds (1). Whereas knowledge about the enzyme systems responsible for the oxidative cleavage of carotenoids and the subsequent formation of polar aroma precursors is still missing, many

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conversion pathways of precursors into important carotenoid-derived aroma compounds could be elucidated (2,3).

Progress in the analysis of polar aroma precursors was strongly influenced by the introduction of suitable isolation and separation techniques. A first breakthrough was achieved by the use of XAD-2 as well as RP-C18 material for the enrichment of polyhydroxylated and glycosidically bound constituents from natural tissues (1,4). For the isolation of precursor compounds from the heterogeneous glycosidic extracts, obtained by the above mentioned solid-phase extraction, a method was required that could cope with large sample loads. Moreover, only very gentle separation conditions could be applied in order not to trigger the aroma formation. For this reason, the all liquid technique of countercurrent chromatography - fulfilling both prerequisites - became a very useful tool for gentle and efficient precursor analysis. In the last years, the application of HPLC coupled with two-dimensional MS using atmospheric pressure ionization has opened new analytical possibilities, because this technique combines the high separation power of HPLC with the possibility of obtaining structural information of trace constituents through mass spectrometric analysis (5, 6).

In the following, the analysis of norisoprenoid aroma precursors of saffron (dried stigmas of *Crocus sativus*), Gardenia fruits (*Gardenia jasminoides*) and of apple leaves (*Malus domestica*) employing countercurrent chromatography as well as HPLC-MS/MS will be reported.

Experimental

Materials. Dried saffron (Crocus sativus), type "electus pulvis" was purchased from A. Galke GmbH, Gittelde, Germany and stored in the dark at -18°C. A methanolic extract from fruits of Gardenia jasminoides was supplied by Professor N. Watanabe, Shizuoka University, Japan. Apple (Malus domestica) leaves were collected in autumn 1996 in Lower Franconia, Germany. All commercial chemicals used were of analytical grade quality. Solvents were redistilled before use.

Extraction of Saffron and Isolation of Glycosidic Constituents. Saffron was successively extracted with petroleum ether, diethyl ether, methanol and water. Part of the methanolic extract was enzymatically hydrolysed using Rohapect D5L (Röhm, Germany) and the released aglycons were analyzed by HRGC-MS. For the initial fractionation of the methanolic extract preparative multilayer coil countercurrent chromatography (MLCCC) was used. The so-obtained subfractions were further fractionated by analytical MLCCC. After acetylation and subsequent flash chromatography, the glycoconjugates were finally purified by normal phase and/or reversed phase HPLC. (For details cf. References 7,8).

Synthesis of 2-Hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one 13 and its Peracetylated Glycoside 13Gac (cf. Figure 4). Starting with isophorone 25 the enol 13 was obtained in 45% yield (10). 13Gac was synthesized using a modified Koenigs-Knorr-reaction with silver trifluoroacetate as catalyst (cf. Reference 11) and purified by preparative HPLC (column: Knauer Vertex with Eurospher 100 5 μ m, 16 x 250 mm, Knauer, Berlin; solvent: 90% MTBE 10% n-pentane; flow rate: 7.5 mL/min; UV-detection: λ =249 nm).

Identification of 2-Glucopyranosyloxy-4,4,6-trimethyl-2,5-cyclohexadien-1one tetraacetate 13Gac Using HPLC-MS/MS. 800 mg of a methanolic extract from saffron was separated into 6 fractions by high speed countercurrent chromatography (HSCCC, Pharma-Tech. Research Corporation; Baltimore, Maryland). The HSCCC apparatus was equipped with 3 preparative coils connected in series (total volume: 850 mL; solvent system: tert-butylmethyl ether/n-butanol/acetonitrile/water (2:2:1:5); revolution speed: 800 rpm; flow rate 5 mL/min; elution mode: head to tail with the dense layer being the mobile phase). The organic solvent was removed under reduced pressure and the remaining aqueous phase was freeze-dried. An aliquot of the fractions was acetylated (Ac₂O/pyridine) at room temperature (2 days). After addition of icewater the acetylated substances were extracted with diethyl ether. The solvent was removed and the residue was dissolved in MeOH and diluted to a concentration of 100 - 200 mg/L. HPLC conditions: LiChroCART® column with LiChrospher® RP-18 5 µm, 4 x 250 mm, Merck, Darmstadt; solvent A: aqueous NH₄Ac (5 mM), solvent B: MeCN; gradient: from 20% MeCN/80% NH₄Ac to 100% MeCN in 30 min, then 10 min 100% MeCN; flow rate: 0.8 mL/min, MS parameters: ionization: APCI, dry gas: 4 L/min, dry temperature: 350°C, nebulizer: 65 psi, APCI temperature: 400°C, HV corona: 2550 V, HV capillary: 4000 V, capillary exit: 60 V, skimmer 1: 20 V, skimmer 2: 10 V, trap drive: 45, Scan range m/z 50 – 1500. For MS/MS experiments a fragmentation amplitude of 0.8 V and an isolation width of 2.0 m/z were used.

Preseparation of Gardenia Fruits using <u>Multilayer Coil Countercurrent</u> <u>Chromatography (MLCCC) and Isolation of Glycosidic Constituents</u>. Fractionation of the methanolic Gardenia extract using MLCCC was carried out as described above for saffron. Sequential MLCCC fractions were pooled into six groups, *i.e.* combined MLCCC fractions I-VI. After acetylation (Ac₂O/pyridine) of fraction II multiple chromatographic steps (flash chromatography, preparative and analytical HPLC) led to the isolation of compound **29** (cf. Figure 6). The identification of compounds **26-28** was achieved after HSCCC separation of the methanolic extract (conditions as described above for the identification of **13Gac** in saffron by HPLC-MS/MS). *cis*-Crocin **27** coeluted with an unknown compound, which - on the basis of its ESI-MS spectrum - was tentatively identified as an iridoid glycoside. At 120 minutes the elution mode was reversed, which led to the elution of crocetin-monogentiobiosyl ester **28**. UV-VIS absorption maxima, ESI-MS/MS data and ¹H-NMR data of **26** and **27** were identical with the respective compounds from saffron. The molecular weight of **28** was determined to be 652 daltons (pseudomolecular ion at m/z 675 $[M(652)+Na]^+$).

Extraction of Apple Leaves. Apple leaves (3.2 kg) were washed with benzene and homogenized in MeOH. The isolate (277.5 g) was diluted with water and subjected to solid phase extraction using XAD-2 resin. After elution with MeOH, concentration and freeze-drying 119.4 g of a glycosidic extract was obtained. For details cf. Reference (12).

Simultaneous Distillation/Extraction (SDE). The composition of the methanolic extract was analyzed by SDE under acidic conditions (pH 2.5) with diethyl ether/pentane (1:1) as organic phase. After careful concentration, the distillate was analyzed by HRGC-MS.

Fermentation Studies. An aliquot of the glycosidic extract was dissolved in H_2O and baker's yeast was added. The suspension was stirred in the absence of air for 5 days. After centrifugation and subsequent liquid-liquid-extraction of free volatiles with diethyl ether, the aqueous residue was subjected to SDE treatment. The generated volatiles were analyzed by HRGC-MS.

Isolation of Compound 33 (cf. Fig. 7) Using High Speed Countercurrent Chromatography (HSCCC). HSCCC was performed in analogy to the saffron analysis by using a revolution speed of 1000 rpm and a flow rate of 4 mL/min. Seven fractions were obtained after TLC-analysis. Riesling acetal 31 and 1,1,6trimethyl-1,2-dihydronaphthalene (TDN) 32 were generated from fractions V and VI after simultaneous distillation/extraction (SDE) under acidic conditions (pH 3). After acetylation, subsequent flash chromatography using a pentane/diethyl ether gradient followed by normal phase HPLC-separation (column: Hypersil 4.6 x 250 mm, Knauer, Berlin) the TDN progenitor 33 was isolated from the combined fractions V and VI.

High-Resolution Gas Chromatography-Mass Spectrometry (HRGC-MS). HRGC-MS was performed with a Hewlett-Packard GCD system equipped with a PTV injector (KAS-system, Gerstel, Mülheim, Germany) and a J&W DB-5 column (30 m x 0.25 mm i.d., film thickness 0.25 μ m). For details cf. Reference (12).

HPLC-MS/MS. An HP Series 1100 binary pump driven by Chemstation chromatography software was connected to a Bruker Esquire Ion Trap Mass Spectrometer using atmospheric pressure chemical ionization (APCI). Dry gas was nitrogen.

Nuclear Magnetic Resonance (NMR). ¹H and ¹³C NMR spectra were recorded on Bruker AMX 300 and AM 400 spectrometers.

Spectroscopic Data for 13. EI-MS, m/z (%): 153 (4); 152 (M⁺, 41); 137 (26); 125 (4); 124 (49); 123 (17); 110 (13); 109 (100); 107 (8); 105 (3); 95 (9); 94 (6); 93 (2); 91 (15); 82 (2); 81 (23); 79 (21); 77 (11); 69 (8); 67 (8); 66 (4); 65 (11); 63 (4); 55 (10); 53 (14); 52 (2); 51 (7); RI (DB-5): 1161; RI (CW): 1721; ¹H-NMR (300 MHz, CDCl₃, δ in ppm): 1.26 (6H, s, CH₃-C4); 1.90 (3H,

d, J = 1.5 Hz, CH₃-C6); 6.07 (1H, d, J = 3.5, H3); 6.29 (1H, s, OH); 6.72 (1H, dq, J = 3.5, 1.5 Hz, H5); ¹³C-NMR (75 MHz, CDCl₃, δ in ppm): 15.4 (CH₃-C6); 27.4 (2 CH₃-C4); 37.2 (C4); 124.9 (C3); 130.8 (C2/C6); 145.4 (C6/2); 155.7 (C5); 182.4 (C1).

Spectroscopic Data for 13Gac. ESI-MS: pseudomolecular ion at m/z 505 [M(482) + Na]⁺; ¹H-NMR (300 MHz, CDCl₃, δ in ppm): 1.88 (3H, d, J = 1.0 Hz, CH₃-C6); 2.02-2.13 (4 x 3H, 4s, 4 CH₃COO); 3.74 (1H, ddd, J = 10.0, 5.0, 2.5 Hz, H5'); 4.15 (1H, dd, J = 12.5, 2.5 Hz, H6a'); 4.21 (1H, dd, J = 12.5, 5.0 Hz, H6b'); 4.99 (1H, d, J = 8.0 Hz, H1'); 5.10 (1H, dd, J = 10.0, 9.0 Hz, H4'); 5.18 (1H, dd, J = 9.5, 8.0 Hz, H2'); 5.28 (1H, dd, J = 9.5, 9.0 Hz, H3'); 6.38 (1H, d, J = 3.0 Hz, H3); 6.57 (1H, dq, J = 3.0, 1.0 Hz, H5); 2 CH₃-C4 obscured.

Spectroscopic Data for 29 (as peracetate). ESI-MS: pseudomolecular ion at m/z 579 [M(556) + Na]⁺; ¹H-NMR (400 MHz, CDCl₃, δ in ppm): 1.19 and 1.22 (6H, 2s, 2 CH₃-C6); 1.57 (3H, s, CH₃-C2); 1.76 (1H, m, H5a); 1.99-2.06 (5 x 3H, 5s, 5 CH₃COO); 2.45 (1H, dd, J = 17.0, 6.0, H3b); 3.86 (1H, m, H5'); 4.13 (1H, dd, J = 12.0, 2.0 Hz, H6a'); 4.29 (1H, dd, J = 12.0, 4.0 Hz, H6b'); 5.07 (1H, m, H4); 5.11-5.22 (2H, m, H2', H4'); 5.26 (1H, dd, J = 9.5, 9.5 Hz, H3'); 5.84 (1H, d, J = 8.0 Hz, H1'). (H3a and H5b obscured by acetate signals).

Results and Discussion

Formation of Norisoprenoid Aroma Compounds

The formation of norisoprenoid aroma precursors as well as of norisoprenoid volatiles has to be regarded as multistep conversion of intact C_{40} -carotenoids that gives rise to a wide variety of secondary metabolites. Since labeling studies are missing, the classification of these compounds as degradation products of carotenoids is still speculative. Support for an apocarotenoid pathway comes from a number of arguments:

- For degradation products the absolute configuration of asymmetric carbon atoms that bear functional groups or the side chain of cyclic apocarotenoids is identical with that of the presumed carotenoid precursor (cf. compounds 4 and 5 in Figure 1).
- ii) In certain cases, complementary fragments, *e.g.* C_{27} and C_{13} -fragments from rose flowers (13) as well as C_{10} and C_{20} -fragments from saffron (14) could be isolated (cf. 2 and 3, 5 and 6 in Figure 1).
- iii) Upon curing/fermentation of tea (15) and tobacco (16) and upon ripening of grapes (17) the level of C_{13} -compounds increases while the level of carotenoids decreases (2).

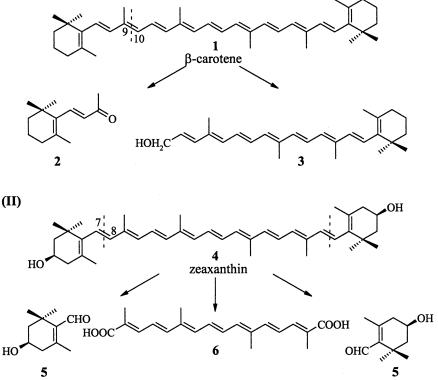


Figure 1. Assumed cleavage of carotenoids in the case of (I) rose flowers and (II) saffron.

Carotenoid degradation is initiated by an oxidative cleavage of the intact carotenoid, which leads to the formation of aldehydes and ketones with shorter chain length. The functional groups are subjected to further oxidation or reduction yielding carboxylic acids or alcohols, respectively (cf. Figure 1). Knowledge about the enzyme systems responsible for the primary oxidative catabolism is sparse and still the topic of current research. The variety of oxidation products found in nature up to now implies that there are some preferred oxidation sites in the molecules as for example the 9,10-double bond giving rise to the large group of C₁₃-norisoprenoids. But there are also various other products formed with a different number of carbon atoms (cf. Reference 2 for an overview). After further enzymatic transformation steps, the primary cleavage products are converted into reactive aroma precursors.

Norisoprenoid aroma precursors can be divided into different classes (cf. Figure 2). By far the largest group are glycosidically bound components. Degradation of the glycoconjugates can be acid-catalyzed, *e.g.* during fruit processing or enzymatically, *e.g.* during fermentation. The glycosylation of a polyol moiety not only results in an increased stability of the precursor but also influences aroma formation directly by favoring certain elimination reactions. This was shown for β -damascenone **30** as well as for vitispirane **11** formation, respectively (*18*). Another class of progenitors are the polyols which - upon (allylic) elimination of water often accompanied by a cyclization step - are transformed into volatiles. An example is the reactive 1,6-allyldiol 7 that under gentle reaction conditions (natural pH, room temperature) is converted into isomeric theaspiranes **10** (*3*). A third class of carotenoid derived aroma precursors are glucose esters, *e.g.* C₁₀-compound **9**, that gives rise to isomeric marmelolactones **12**, key aroma constituents of quince fruit (*19*).

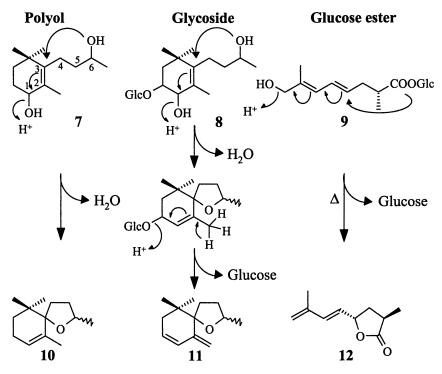


Figure 2. Examples for the formation of norisoprenoid aroma compounds from different classes of reactive progenitors, i.e. polyols, glycosides and glucose esters (for details cf. text).

Analysis of Norisoprenoid Aroma Compounds

A general work-up scheme for the analysis of glycosylated aroma precursors has been published (18). It is applicable for polyhydroxylated constituents as well. An enrichment of the target compounds is achieved using solid phase extraction (XAD-2 or RP-C18 material). For the initial separation of the resulting extract, countercurrent chromatography was found to be most helpful. In a single run, separation in the gram range is possible by using inexpensive solvent mixtures instead of expensive solid phase materials. The absence of any solid packing material not only saves money, but also avoids adsorption losses and the formation of artifacts. Thus, a total recovery of the sample is guaranteed.

One of the most widely used CCC technique is <u>multilayer coil countercurrent chromatography</u> (MLCCC), which is also called HSCCC (<u>high speed countercurrent chromatography</u>). It was introduced in 1981 by Ito (20). The "chromatographic column" is a long teflon tubing (80-150 m, volume 50 – 850 mL) that is wrapped around a holder in several layers. This so-called 'multi-layer coil' contains a biphasic solvent mixture, which – due to the rotation of the coil in a planetary system – is exposed to a fast changing centrifugal force field. Thus, a continuous mixing and settling of the two phases takes place providing a highly efficient partitioning of the analytes. For detailed description of the technique (20) as well as applications (5,21-23) refer to the references cited.

A different approach in norisoprenoid aroma precursor analysis is the application of HPLC-MS/MS in connection with soft atmospheric pressure ionization techniques. When an authentic specimen of the target compound is available, a screening for this substance can be performed even in crude extracts. A helpful method is <u>selected reaction monitoring</u> (SRM), where the specificity and sensitivity of the MS analysis is enhanced by focussing on a substancecharacteristic ion and its relationship to certain daughter ions. Thus, suppression of the majority of background signals and the detection of compounds even without a complete separation can be achieved (6). HPLC-MS/MS offers the advantages of providing structural information without the need of isolating a target molecule.

Examples of Aroma Precursor Studies

Saffron (Crocus sativus)

GC-MS analysis of the combined petrol ether and diethyl ether extracts of dried saffron revealed the presence of a variety of carotenoid-derived aroma compounds. Besides the major constituent, i.e. safranal 14, the following volatiles could be identified through comparison of their chromatographic and mass spectral data with those of authentic reference compounds (for numbering cf. Figure 3 and 4): 3,5,5-trimethyl-2-cyclohexen-1-one (isophorone, 25), 2,6,6trimethyl-2-cyclohexen-1-one, 2,6,6-trimethyl-2-cyclohexene-1,4-dione, 2,6,6trimethylcyclohexane-1,4-dione, 2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one 13, 2-hydroxy-3,5,5-trimethyl-2-cyclohexene-1,4-dione 15, 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one 17, 4-hydroxy-3-oxo-2,6,6-trimethyl-1,4cyclohexadiene-1-carboxaldehyde 19, 5-hydroxymethyl-4,4,6-trimethyl-7-oxa-4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1bicyclo[4.1.0]heptan-2-one 20, carboxaldehyde 21, 4-hydroxymethyl-3,5,5-trimethyl-2-cyclohexen-1-one 22, and 3-hydroxy- α -ionol 24 (24). Sensory studies revealed that besides safranal 14 ('saffron-like') additional compounds contribute to saffron aroma (25,26). Cadwallader and coworkers identified 2-hydroxy-4,4,6-trimethyl-2,5cyclohexadien-1-one 13 ('saffron-like, dried hay note') as an additional key flavor compound (26).

In order to elucidate the generation of saffron aroma, a methanolic extract of the spice was prepared. The GC-MS analysis of the enzymatically liberated aglycons is shown in Figure 3. On the basis of this screening, the presence of numerous glycosidic aroma precursors in saffron was apparent. Compounds identified included 2-hydroxy-4,4,6-trimethyl-2,5positively inter alia cyclohexadien-1-one 13, safranal 14, 2-hydroxy-3,5,5-trimethyl-2-cyclohexene-1,4-dione 15, 4-hydroxy-2,6,6-trimethyl-2-cyclohexen-1-one 16, 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one 17, 4-hydroxy-3-oxo-2,6,6-trimethyl-1cyclohexene-1-carboxaldehyde 18, 4-hydroxy-3-oxo-2,6,6-trimethyl-1,4-cyclo-5-hydroxymethyl-4,4,6-trimethyl-7-oxa-bihexadiene-1-carboxaldehyde 19, cyclo[4.1.0]heptan-2-one 20, 4-hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde 21, 4-hydroxymethyl-3,5,5-trimethyl-2-cyclohexen-1-one 22. 6-hydroxy-3-hydroxymethyl-2,4,4-trimethyl-2,5-cyclohexadien-1-one 23, and 3-hydroxy- α -ionol 24.

The methanolic extract containing the intact glycosides was subjected to MLCCC without further enrichment. MLCCC subfractions were acetylated and finally purified by HPLC. Thus, a considerable number of glycosidic constituents could be isolated and structurally characterized. The structures of novel carotenoid-derived metabolites that have been identified for the first time in saffron have been published elsewhere (7, 8, 27).

Since for the odor-active hydroxyketone 13, no glycosidically bound precursor could be isolated, we decided to synthesize its likely precursor, i.e. 2glucopyranosyloxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one 13G. Synthesis of the aglycon moiety was performed according to Ref. (10), starting with isophorone 25 (cf. Figure 4). In a modified Koenigs-Knorr synthesis (11) the peracetylated glycoconjugate 13Gac was obtained. This reference compound enabled the identification of precursor 13Gac in the acetylated saffron extract (cf. Figure 5). As ionization method APCI in the positive mode was found to be most sensitive employing a gradient of MeCN/NH₄Ac.

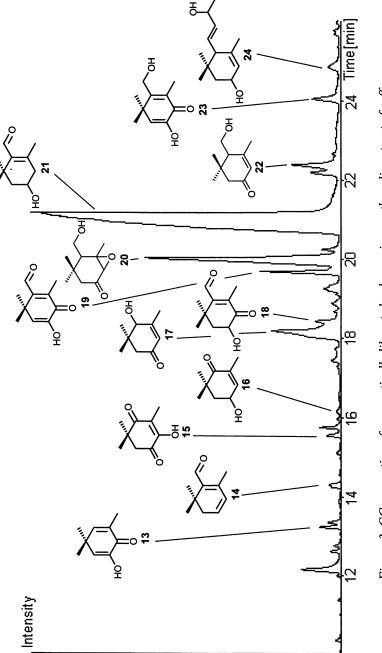


Figure 3. GC separation of enzymatically liberated aglycons in a methanolic extract of saffron (for details cf. Experimental and Ref.12)

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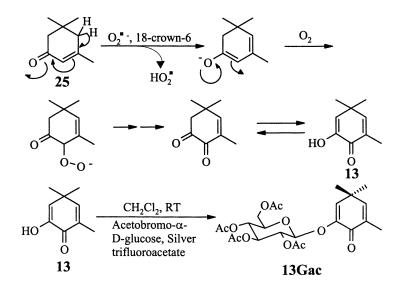


Figure 4. Synthesis of the peracetylated glycoside of 2-hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one **13Gac.**

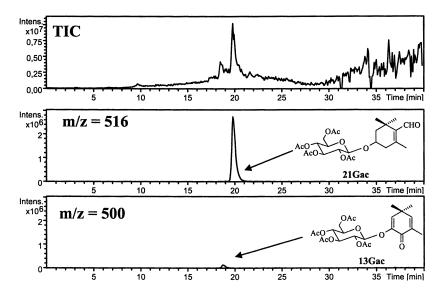


Figure 5. HPLC-MS/MS analysis of fraction 4 of the acetylated saffron extract (HPLC and MS conditions cf. Experimental, m/z = 500/516 same scale).

Acetylated picrocrocin **21Gac** as well as the precursor compound **13Gac** were identified as ammonium adducts in the respective ion chromatograms of CCC fraction 4 (mass track m/z 516 and m/z 500 in Figure 5). By comparison of the retention times and the main fragment ions in SRM experiments with the reference compounds, the assignment of the peaks could be verified. This demonstrated the advantages of HPLC-MS/MS in the analysis of trace amounts of natural products.

Gardenia (Gardenia jasminoides) fruits

Fruits of *G. jasminoides* are known to contain the same carotenoid-derived pigments as saffron (cf. Figure 6). Thus a search for possible additional carotenoid metabolites, *e.g.* the aroma progenitors outlined in Fig. 3, was carried out. A methanolic extract was prepared in the same manner as in the saffron study. Prefractionation was done by MLCCC and the fractions were purified by preparative and analytical HPLC. The study is not yet completed, but up to now besides glycosidically bound iridoids, only three crocetin derivatives **26-28** and the novel C_{10} -carotenoid-derived glucose ester **29** could be isolated. This analysis shows that the quantity and quality of the aroma precursor not only depends on the carotenoid pattern but also on the enzymatic activities present in the plant as well as the treatment of the plant material.

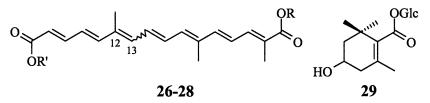
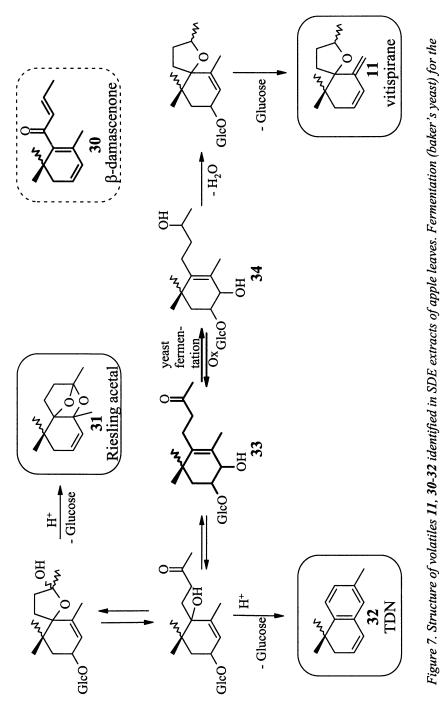


Figure 6. Carotenoid-derived compounds isolated from Gardenia fruits: 12,13-trans, R=R'=gentiobiosyl: Crocin 26; 12,13-cis, $\hat{R}=R'=gentiobiosyl$: 13-cis-Crocin 27; 12,13-trans, R=H, R'=Gentiobiose: Crocetin-monogentiobiosyl ester 28; 4-hydroxy-2,2,6-trimethyl-1-cyclohexene-1-carboxy acid glucose ester 29; Glc: Glucose.

Apple (Malus domestica) leaves

Apple leaves are known to generate highly attractive aroma compounds, such as the intensely odorous β -damascenone **30** (cf. Figures 7 and 8). Since β -damascenone is in common use as flavorant (28,29), additional natural sources for the production of this key flavor compound are still sought after.

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conversion of TDN-progenitor 33 into vitispirane precursor 34 (for details cf. text).

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This prompted us to investigate the potential of apple leaves for the production of flavor compounds. In a first step, the aroma release from apple leaves was studied by SDE. The aroma-active C_{13} -norisoprenoids vitispirane 11, Riesling acetal 31, β -damascenone 30 (500 µg/100 g leaves) and TDN 32 could be detected after heat-treatment under acidic conditions (SDE pH=2.5). Whereas ketone 30 as well as norisoprenoids 11 and 31 possess pleasant aromas, TDN 32 exhibits an undesired "kerosene-like" note (30). Hence a reduction of the TDN content is necessary before apple leaves can be used as a flavor source.

In order to identify possible TDN-precursors in apple leaves, we focussed on the known TDN-progenitor 3,4-dihydroxy-7,8-dihydro-\beta-ionone \beta-D-glucopyranoside 33. Glucoside 33 was previously isolated by our group from Riesling vine leaves (30, cf. Figure 7). After MLCCC fractionation of the glycoside extract, the fractions were screened by TLC using the authentic reference compound 33. This enabled the quick isolation of 33 from the apple leaf extract by analytical HPLC. Glucoside 33 is structurally related to glucoconjugate 34, which can be converted into the flavor compounds vitispiranes 11 (3). Hence, it was attempted to enhance vitispirane formation through reduction of 33 with baker's yeast. By fermentation of the glycosidic apple leaf extract, the amount of thermally generated TDN 32 could be reduced by almost 20 %, and the concentration of the desired vitispiranes 11 increased from 30 to 72 %. It is noteworthy that the amount of β -damascenone 30 was also slightly reduced. Due to the low flavor threshold (0.002 ppb) (31) the aroma impact of ketone 30 was still significant. This example shows that knowledge about the structure of aroma precursors is helpful for successfully applying biotechnological conversions. This could gain importance for other aroma releases as well, e.g. within the scope of winemaking. By selection of appropriate wine yeasts, it seems to be possible to control certain reaction sequences and to reduce the risk of TDN formation during the aging of wines.

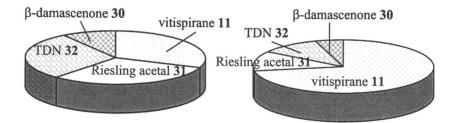


Figure 8. Aroma profiles obtained by SDE of a glycosidic extract from apple leaves; left: before fermentation, right: after fermentation with baker's yeast.

Summary

It has been shown that for the isolation of norisoprenoid progenitors solid phase extraction using either XAD-2 or RP-C18 material in conjunction with preparative scale separation of the isolate using countercurrent chromatography is still the method of choice. The isolation of authentic reference samples is a crucial step in aroma precursor analysis for two reasons: first it sheds light on aroma formation in plants from polar precursors through comparison of the bound and free fraction and, secondly, it enables a quick screening for certain compounds in different plant sources using the sensitive and highly specific technique of HPLC-MS/MS.

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Chapter 3

HPLC-MS Analysis of Carotenoid-Derived Aroma Precursors

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A number of carotenoid-derived aroma compounds exhibit exceptionally attractive sensory properties and low odor thresholds. Considerable efforts have been achieved during the last decades concerning the structural elucidation of precursors involved in the generation of carotenoid-derived flavor substances. However, besides the identification of a number of glycosidically bound intermediates and oxygenated progenitors, our knowledge about the biochemical pathways of carotenoid catabolism is still limited. In order to gain a better understanding about intermediates involved in both the biosynthesis and degradation of carotenoids, this paper summarizes experimental concepts and analytical methods based on the application of HPLC coupled to tandem mass spectrometry (MS/MS) by means of electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI). Furthermore, recent results from HPLC-MS/MS analyses of C_{40} -carotenoids, C_{20} -retinoids, and from studies on polar metabolites of the methylerythritol phosphate pathway of terpene biosynthesis are presented.

During the last decades substantial progress has been accomplished with respect to the structural elucidation of carotenoid related aroma compounds and their specific precursors (1). However, the relevant information collected mainly has focused on the identity of polyhydroxylated progenitors - so-called polyols -

and glycoconjugates of trimethylcyclohexenyl derivatives such as safranal or β ionol. Our knowledge about the biochemical reactions responsible for selective carotenoid degradation is still limited and the identity of many key intermediates remains to be established (2). An overview on putative pathways for the formation of carotenoid derived flavor compounds is shown in Figure 1.

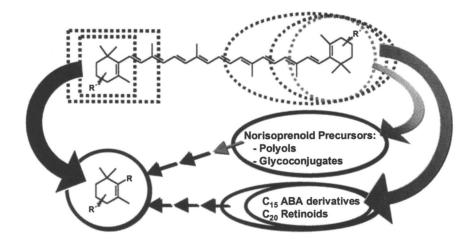


Figure 1. Putative biochemical pathways involved in the formation of carotenoid derived flavor compounds (for details cf. text).

Cleavage of the carotenoid chain by dioxygenases is expected to yield prominent flavor compounds such as the C_{10} -metabolite safranal (oxidative cleavage of the 7,8 or 7',8' double bonds followed by elimination of water from C-3) or the C_{13} -norisoprenoid β -ionone (oxidative cleavage of the 9,10 or 9',10'double bonds). While little is known about such enzyme systems in higher plants, the β -carotene dioxygenase giving rise to C_{20} -retinoids recently has been cloned from *Drosophila melanogaster* (3). Accordingly, the corresponding 9*cis*-epoxycarotenoid dioxygenase responsible for the formation of abscisic acid (ABA; cleavage of the 11,12 double bond of 9-*cis*-violaxanthin or 9'-*cis*neoxanthin) has been characterized in maize as well as in leaves of *Phaseolus vulgaris* (4, 5). It is worth to note, that at least in fungi ABA can also be generated by *de-novo* biosynthesis (6).

Besides by the direct catabolic breakdown of C_{40} -carotenoids, C_{10} to C_{13} flavor compounds can also be formed by β -oxidation or Baeyer-Villiger-type

reactions from smaller terpenoids such as C_{15} -ABA derivatives or β -ionone (7, 8). In addition, the oxidative degradation of C_{20} -retinoids has been described to yield such aroma substances (2 and references cited therein, 9-11). Finally, hydrophilic oxygenated compounds - so-called 'polyols' - represent a third class of progenitors. In many plant tissues such flavorless intermediates accumulate as glycosides (2,12) or are further metabolized to yield sulfates (13). Prominent examples include the precursors of key flavor compounds like edulans, theaspiranes, vitispiranes, β -damascenone, marmelo oxides and marmelo lactones (14-18).

Analysis of Carotenoid-derived Aroma Precursors

Most of the carotenoid breakdown products shown in Figure 1 are rather polar and labile compounds which excludes a direct analysis by GC-MS. For screening purposes, biomimetic acid-catalyzed breakdown by simultaneous distillation and extraction (SDE) can liberate the volatile flavor compounds. This approach was extensively applied to the study of carotenoid-derived aroma precursors (16). However, complex transformations - i.e. cleavage, reduction, dehydration and rearrangement reactions - are commonly required to yield the volatile carotenoid-derived flavor substances. Hence, SDE followed by GC-MS often provides only limited structural information about the relevant precursors. Accordingly, derivatization of polyols and their glycoconjugates bv peracetylation or permethylation reactions yields stable and volatile products which can be characterized by GC-MS. Again, any chemical modification interferes with the reactivity of the carotenoid-derived aroma precursors and can encumber detection of the free volatiles by subsequent SDE and GC-MS experiments.

Consequently, studies focusing on the identification of genuine carotenoidderived aroma precursors largely rely on analytical methodology based on high performance liquid chromatography. For preparative purposes, separations by means of countercurrent chromatography techniques have been successfully applied in addition to conventional liquid chromatography (19). The following chapter summarizes experimental strategies and methods for the analysis of carotenoids and their metabolites based on the application of atmospheric pressure ionization mass spectrometry coupled to HPLC. Hence, we survey studies focused on the identification of polar norisoprenoidal precursors and oxygenated retinoids and discuss problems related to the ionization of carotenoids by means of the electrospray process.

HPLC-MS Analysis of Carotenoids and Xanthophylls

A variety of carotenoids and xanthophylls serves as ultimate precursors during the formation of the corresponding aroma substances. To establish their identity, chromatographic separation is usually achieved by reversed phase HPLC based on the application of C_{18} or C_{30} -modified stationary phases. For a comprehensive overview on extraction and saponification techniques, as well as a review of numerous HPLC methods for the chromatographic determination of carotenoids in food, the interested reader is referred to ref. (20).

Following separation, structures of many new carotenoids and carotenoid conjugates have been established over the last decades with the aid of mass spectrometry (21, and references cited therein). Besides accurate mass measurements to determine the molecular formula for the individual carotenoid, diagnostic fragment ions of structural significance such as ions m/z 165 and m/z 205 generated by electron impact ionization (EI) of 5,6- and 5,8-epoxides have been identified. In order to facilitate identification of the molecular ions M^+ or [M+H]⁺, soft ionization techniques like chemical ionization (CI), fast atom bombardment (FAB) and matrix-assisted laser desorption ionization (MALDI) have been applied (21-25). To obtain more structural information, the FAB source was coupled to a sector field tandem mass spectrometer (FAB-MS/MS) and MALDI was combined with post-source-decay time-of-flight (PSD-TOF) mass spectrometry (26). However due to instrumental constraints, application of HPLC coupled directly to mass spectrometry was limited to a few examples based on the continuous-flow FAB technique (22, 23).

The development of techniques utilizing atmospheric pressure ionization, namely electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), represents a major experimental break-through and was essential for the successful coupling of HPLC with mass spectrometry in recent years (27,28). While ESI and APCI yield molecular ions of most analytes, the combination of these soft ionization techniques with tandem mass spectrometry provides a tool to identify established natural products and to elucidate structures of hitherto unknown compounds in complex matrices.

With respect to carotenoids, van Breemen (29) reported the first analytical method based on HPLC-ESI-MS in 1995. Since then, numerous applications of both ESI (30-32) and APCI (33-34) have been published. Still, some critical experimental constraints related to the ESI analysis of non-polar carotenoids remained to be solved: According to the nature of the electrospray process, the analytes are *normally* not directly ionized. Instead ESI merely assists the effective transfer of preformed analyte ions from solution into the gas phase (35). However, under standard working conditions for ESI, the formation of protonated or deprotonated molecular ions of carotenoids and retinoids is not likely to occur due to an obvious lack of basic or acidic functional groups.

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Consequently, for successful electrospray ionization three different experimental concepts proved to be feasible: (i) The generation of molecular ions M^+ from carotenoids by electrochemical one-electron oxidation in the ESI interface which can be mediated by postcolumn addition of halogenated solvents such as heptafluoro-1-butanol (29). (ii) The generation of molecular cations M^{2+} by carotenoid oxidation in presence of SbF₅ (36), and (iii) the formation of adduct ions $[M+Ag]^+$ following post-column addition of silver perchlorate AgClO₄ to the solvent (31,32).

In order to provide an analytical basis for the identification by HPLC-ESI-MS/MS of complex mixtures of carotenoids and xanthophylls, many of which are structurally closely related, we aimed to avoid postcolumn addition of reagents and solvents. Mixing of the strong oxidant SbF₅ directly to the solvent system (i.e. HPLC analysis with SbF₅ containing eluents) was impracticable due to problems related to the chemical stability of the tubing and packing materials used for the HPLC columns. Likewise, application of eluents like heptafluoro-1butanol was expected to interfere with established protocols for chromatographic separation of carotenoids and was therefore not attractive from an economical point of view. Consequently, we studied ionization of carotenoids by the electrospray process following addition of Ag⁺ reagents. It was attempted not only to detect carotenoids by their $[M+Ag]^+$ molecular ions, but also to examine the influence of position and configuration of double bonds on the respective product ion spectra obtained from these $[M+Ag]^+$ precursor ions.

ESI-MS Analysis of Carotenoids: Experimental Design and Results

Standard solutions of the respective carotenoid (1 mg/ml, acetone) were prepared from β -carotene, lycopene, lutein, zeaxanthin and capsanthin. Samples were diluted to a final concentration of 10 µg/ml in acetone-water (9/1 v/v). To induce formation of [M+Ag]⁺ molecular ions, aqueous solutions of AgNO₃ or AgClO₄ were added prior to the analysis to achieve a final concentration of 5 µmol/ml Ag⁺. 5 µl of the sample were then directly introduced into the mass spectrometer (Finnigan TSQ 7000) with acetonitrile (50 µl/min) as carrier. Typical ESI mass spectra of β -carotene are shown in Figures 2A/2B.

Without Ag^+ addition as well as in the presence of $AgNO_3$ all carotenoids under investigation were detected as their molecular radical cation M^+ except the oxo-derivative capsanthin which yielded solely the protonated molecule $[M+H]^+$. Efficiency of the ESI process was quite good with full scan ESI mass spectra (S/N better than 100) recorded following injection of approximately 100 pmol β -carotene in the presence of 25 nmol AgNO₃. Signals at *m/z* 553.7 in Figure 2A apparently represented oxidized degradation products of β -carotene which were obviously present in the sample prior to AgNO₃ addition. Under no circumstances could $[M+Ag]^+$ adducts be detected following addition of either AgNO₃ or AgClO₄. As shown in Figure 2B, upon AgClO₄ addition β -carotene, lutein and zeaxanthin yielded "[M-H]⁺" molecular ions together with newly formed oxidized derivatives M'.

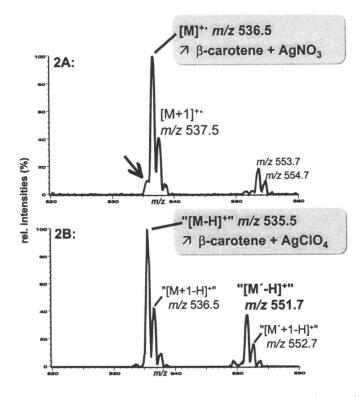


Figure 2. ESI-MS spectrum of β -carotene in the presence of 5 μ mol/ml AgNO₃ (2A) or AgClO₄ (2B).

ESI-MS Analysis of Carotenoids: Discussion

Our results clearly demonstrate the influence of the experimental conditions and, presumably, of the design of the ESI interface on the ESI mass spectra obtained for carotenoids. Most important, we utilized an ion source, in which the high voltage (3 - 6 kV) was directly applied to the ESI spray capillary made from stainless steel. Accordingly, as result of the electrolytic nature of the electrospray process (36,37), we detected molecular ions M^+ most likely formed by anodic one-electron oxidation of the carotenoids (29) in presence of AgNO₃ which served as electrolyte (Figure 2A). However, one should note that discharge reactions at the capillary tip are under discussion to trigger gas-phase ionization mechanisms, which as well might be responsible for the formation of radical cations (37). While in the presence of AgNO₃ only minor amounts of ions m/z 535.5 could be detected (indicated by the arrow in Figure 2A), addition of the strong oxidant AgClO₄ resulted in the exclusive formation of formal "[M-H]⁺" molecular ions m/z 535.5 (Figure 2B). In presence of AgClO₄ any significant generation of M⁺ species could be excluded according to the relative intensity of the ion m/z 536.5 which represents the ¹³C-isotopomer of the [M-H]⁺ molecular ion rather than the radical cation M⁺. From a mechanistic point of view, electrochemical studies applying cyclic voltametry already have shown, that two-electron oxidation of β -carotene initially yielded the cation M²⁺, that has a rather short half-life of less than 6 s (*36*). However, such cations were not visible during our studies following the addition of AgClO₄. Instead, the β carotene²⁺ cation obviously was involved in a successive chemical reaction which resulted in the loss of a proton. As result, the [M²⁺-H⁺]⁺ molecular ion m/z 535.5 was observed in Figure 2B as base peak owing to this combined twoelectron oxidation plus chemical reaction mechanism.

The identity of molecular ions m/z 535.5 and m/z 536.5 was confirmed by the product ion spectra shown in Figure 3. Interestingly, these spectra were generated by collisional induced dissociation of precursor ions applying rather low collision energies. Nevertheless, they closely resembled the well-known EI mass spectra. As anticipated, in-chain fragmentation of β -carotene resulted in the neutral loss of toluene (Δ 92 amu; m/z 444 or m/z 443) and xylene (Δ 106 amu; m/z 430 or m/z 429). Furthermore, the intensity ratio R [I_{M-92}/I_{M-106}] of product ions generated by collision induced dissociation (R=10 for M⁺), the absence of any [M-56 amu] cations, and the loss of 79 amu from precursor ion m/z 535 (product ion m/z 456), which was presumably formed via a sevenmember transition state, has been observed in the EI mass spectra of β -carotene before and confirmed identity of molecular ions M⁺ and [M²⁺-H⁺]⁺(21).

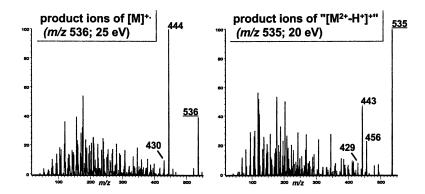


Figure 3. Product ion spectra of β -carotene confirming identity of M^{+} and $[M^{2+}-M^{+}]^{+}$ molecular ions.

Identity of the oxidized carotenoid M' in Figure 2B (m/z 551.7 [M'²⁺-H⁺]⁺), which was generated in the presence of silver perchlorate, could be established by the product ion spectrum shown in Figure 4: Neutral loss of 80, 92, 106 and 146 amu together with the highly diagnostic product ion m/z 205 demonstrated formation of the corresponding 5,6- or 5,8-epoxide of β -carotene (21). Notably, after extending the incubation time to 60 min prior to the ESI-MS experiments, oxidized carotenoids were the exclusive products in presence of AgClO₄.

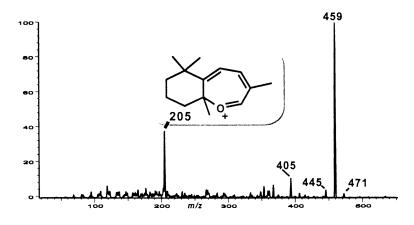


Figure 4. Product ion spectrum of precursor ion m/z 551.7 $[M'^{2+}-H^+]^+$.

Finally, by unequivocal detection of silver clusters $[2 \text{ MeCN}+\text{Ag}]^+$ at m/z 189 and m/z 191 as well as $[3 \text{ MeCN}+\text{Ag}]^+$ at m/z 230 and m/z 232 (characteristic abundance of natural isotopes ${}^{107}\text{Ag}$: ${}^{109}\text{Ag} = 52\%$: 48%) it was demonstrated that we were in principle capable to detect Ag⁺ adducts (data not shown). The failure to identify any Ag⁺ adducts of carotenoids in presence of a surplus of Ag⁺ can be explained by the following reasons:

- (i) Differences in interface design and by tuning of interface parameters: The reported detection of Ag^+ adducts was performed with an ESI interface where the desolvation process for the molecular ions was assisted by passing a curtain of N₂ drying gas (31,32). The Finnigan ESI source used in the present study applied an 20 cm long heated steel capillary as inlet system, where interaction of analyte ions with the extended surface of the capillary may occur and collision of cluster ions with other vaporized molecules could result in degradation of labile Ag^+ clusters as well.
- (ii) Solvent system: The solvent system used was slightly modified compared to the conditions reported in ref. (32); still the published method for the detection of Ag⁺ adducts relied on water-acetone eluents as well.

(iii) Other factors: Most conceivable, the predominant formation of molecular ions M⁺ and [M²⁺ - H⁺]⁺ made the adduct formation of already charged carotenoids with Ag⁺ less likely to occur due to coulombic repulsion effects.

Our experiments show that HPLC-ESI-MS/MS provides a valuable analytical tool for the identification of complex mixtures of carotenoids acting as precursors for the formation of norisoprenoidal flavor substances. "Conventional" ESI proved to be the method of choice for carotenoid analysis with ESI interface designs where the high voltage required to generate the spray was applied directly to the stainless steel spray capillary. Under these experimental conditions, the ESI spray capillary served as electrolytic reaction chamber and addition of trace amounts of electrolytes such as AgNO₃ could increase yields of molecular ions M^+ formed by one-electron oxidation from carotenoids. In contrast, addition of AgClO₄ was less favorable because it yielded - depending on reaction conditions - changing amounts of M^{2+} , $[M^{2+}-H^+]^+$, and oxidized $[(M+O)^{2+}-H^+]^+$ derivatives, respectively.

HPLC-MS Analysis of Oxygenated Retinoids

As outlined in Figure 1, apo-carotenoids, retinoids and abscisic acid derivatives have to be taken into consideration as likely intermediates during the formation of carotenoid derived flavor substances. Again, with the exception of (derivatized) C15 compounds, oxygenated retinoids represent polar and nonvolatile analytes that require methods based on liquid chromatography for their identification. To study the formation of carotenoid related flavor substances, we developed model systems for the degradation of retinol by lipoxygenase catalyzed co-oxidation (38) and by chemical oxidation mediated through mesotetraphenylporphinato-iron(III) in presence of H_2O_2 (39). Besides established volatile products such as β-cyclocitral, dihydroactinidiolide, β-ionone and 5,6epoxy-\beta-ionone, these biomimetic degradation experiments yielded complex mixtures of oxygenated retinoids which had to be analyzed by means of HPLC-MS/MS. Trials to ionize reference compounds demonstrated, that only retinal featuring an aldehyde moiety could be ionized effectively by ESI. In contrast, compounds such as retinol, retinoic acid and the C₂₄-apocarotenoid methylester bixin required APCI for successful HPLC-MS analysis. Following careful optimization of interface parameters, almost exclusively molecular ions were generated by APCI. Degradation reactions such as loss of water were negligible (Figure 5).

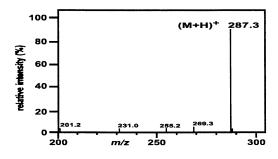


Figure 5. APCI mass spectrum of retinol (vaporizer 300° C, MeOH-H₂O).

HPLC analysis (RP-18, MeOH- H_2O gradient elution) of oxygenated retinoids with UV-detection at 330 nm yielded the chromatogram shown in Figure 6 (39). From the UV spectra it was apparent, that peaks 1, 2 and 4 represented mixtures of polyenes while by comparison with reference compounds peaks 3 and 5 could be identified as 3-dehydroretinol and anhydroretinol.

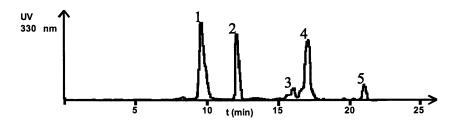


Figure 6. HPLC analysis of oxygenated retinoids.

HPLC-APCI-MS analysis of peak 1 yielded molecular ions $[M+H]^+$ m/z 301 and m/z 303 characteristic for oxo- and hydroxyretinols, respectively. The product ion spectrum of m/z 301 (10 eV; 2 mTorr Ar), which proved to be 4-oxoretinol, revealed the base peak m/z 283 due to facile loss of water and a minor signal at m/z 265 resulting from keto-enol rearrangement combined with the loss of a second water molecule. Also the product ion spectrum of co-eluting m/z 303 shown in Figure 7 demonstrated consecutive neutral loss of two molecules of water. In addition, the product ion m/z 211 of the trimethylbenzene derivative C₁₆H₁₉⁺ established identity of the co-eluting compound as 4hydroxyretinol.

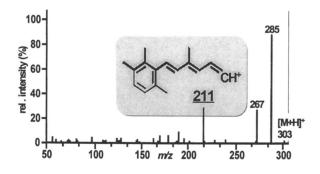


Figure 7. Product ion spectrum of 4-hydroxyretinol (10 eV; 2 mTorr Ar).

The product ion spectra of retinol and *retro*-retinol (Figure 8) showed the anticipated neutral loss of water at m/z 269 and the diagnostic ion m/z 213, which, by comparison with ion m/z 211 of 4-hydroxyretinol, apparently represented the corresponding trimethylcyclohexadienyl cation $C_{16}H_{21}^+$.

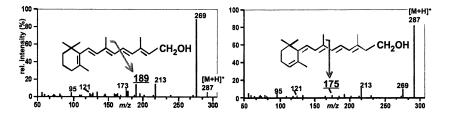


Figure 8. Product ion spectra (12 eV; 2 mTorr Ar) of retinol and retro-retinol.

In order to identify isobaric retinol derivatives, one likely could take advantage of the characteristic ratio of intensities m/z 269 : m/z 213. Yet, this approach required pure reference compounds or, at least, compounds clearly separated by means of HPLC. Consequently, differentiation between coeluting retinol and *retro*-retinol relied on cations $C_{13}H_{19}^+$ m/z 175 formed from *retro*-retinol and $C_{14}H_{21}^+$ m/z 189 derived from retinol (Figure 9).

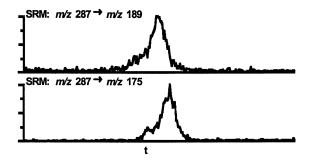


Figure 9. HPLC-MS/MS identification of retinol (m/z 189) and retro-retinol (m/z 175) co-eluting in peak 4 (cf. Figure 6).

Finally, product ion spectra of 5,6- and 5,8-epoxyretinol co-eluting under peak 2 were almost identical. Hence, HPLC-APCI-MS/MS analysis demonstrated the presence of these epoxides, but identification of the individual isomers had to be based on their UV spectra and comparison with data of authentic reference material.

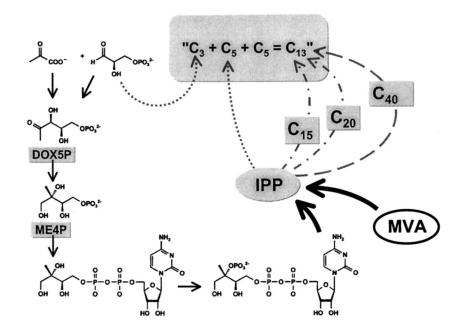
Summarizing our studies on identification of oxygenated retinoids by HPLC-MS/MS, we demonstrated the potential of APCI for generating molecular ions from non-ionic metabolites, which likely are involved in formation of carotenoid derived flavor substances. Still, it became clear that one has to consider all analytical information available (i.e. retention time and UV spectra plus molecular ions and product ion spectra) to ensure a reliable identification of co-eluting oxygenated retinoids.

HPLC-MS Analysis of Metabolites Involved in Biosynthesis of Isoprenoids

Historically, the pathway of terpenoid biosynthesis in animal and yeast cells was established to start with formation of mevalonate (MVA) from three molecules of acetyl CoA (40). As result of subsequent decarboxylation, dehydrogenation and phosphorylation **MVA** steps, yields isopentenyl pyrophosphate (IPP), which together with its isomeric dimethylallyl pyrophosphate (DMAPP) serves as universal precursor for terpene biosynthesis. Rohmer and coworkers, as well as Arigoni and coworkers recently demonstrated in independent studies, that in certain eubacteria and plants some terpenoids are biochemically synthesized via an alternative pathway (41-43).

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Subsequently it was established that 1-deoxyxylulose 5-phosphate (DOX5P), formed by condensation of glyceraldehyde 3-phosphate with pyruvate, represents the first key metabolite of this novel pathway of terpenoid biosynthesis. Further research showed conversion of DOX5P by a reductoisomerase into 2-C-methylerythritol 4-phosphate (ME4P, 44-47), which is subsequently transformed into the respective cytidyl pyrophosphate derivative (ME-CDP, 48). Following a second phosphorylation step modifying the hydroxyl group at C-2 (49), ME-CDP is converted into IPP by enzymatic reactions, which yet have to be clarified (Figure 10).



Most important, in higher plants the enzymes of the MVA pathway are localized in the cytosolic compartment and count responsible for biosynthesis of isoprenoids such as sterols. In contrast, the ME4P pathway (synonyms: Rohmer pathway, DOX5P pathway) operates in the plastids and exclusively supplies the IPP for biosynthesis of all carotenoids, chlorophylls and some sesquiterpenes (50, 51). Consequently, formation of C_{40} -derived norisoprenoids also should involve ME4P as key intermediate. With regard to further isoprenoids that may serve as metabolites for the biosynthesis of norisoprenoid flavor compounds, abscisic acid (ABA) has been demonstrated to be formed directly from ¹⁴C-MVA by the fungi Cercospora rosicola and C. cruenta (for review see refs. 6, 52). This reaction is likely to proceed via farnesyl pyrophosphate (FPP) and inhibitors of carotenoid biosynthesis do not affect ABA accumulation in C. cruenta (53). However, in higher plants such as maize and wilted bean, formation of ABA from ME4P-derived xanthophylls like 9-cisviolaxanthin and 9'-cis-neoxanthin has been studied in great detail (4,5). In avocado fruit, application of the ¹⁴C-labeled precursors MVA and pyruvate demonstrated that ABA is synthesized via both, the classical MVA-route as well as the recently discovered ME4P pathway (54, 55). Finally, studies on the symbiosis between arbuscular mycorrhizal fungi and the roots of terrestrial plants demonstrated that the ME4P pathway is active in plant roots where it can be induced by the fungi. Typical for such arbuscular mycorrhizas is the accumulation of glycosylated C13-norisoprenoids and C14-apocarotenoidal conjugates (56). Most interestingly, growth of barley plants which had been inoculated with the fungus Glomus intraradices in presence of 1-13C-glucose yielded the glycoconjugate of labeled blumenol C (i.e. $3-\infty -7.8$ -dihydro- α ionone). Detailed analysis of the labeling pattern by ¹³C-NMR spectroscopy (Figure 11a-c) demonstrated that the biosynthesis of this particular norisoprenoid proceeded specifically via the ME4P pathway (57). Yet, it has to be clarified, whether blumenol C is formed in such arbuscular mycorrhizas directly, or via the degradation of carotenoid precursors. The incorporation of ¹³C from U-¹³C₆-glucose confirmed the relevance of the ME4P pathway for biosynthesis of blumenol C (57). Still it was a striking observation, that C-9 and C-10 of the hydroxybutyl side chain derived from glucose via ME4P. But according to the characteristic abundance of the ¹³C-isotopomers in the respective C-C units as determined by NMR spectroscopy both C-9 and C-10 might be the remainder of a separate precursor. Consequently, this study represented the first experimental evidence for the existence of a putative $C_3 + (C_5 + C_5)$ pathway involved in biosynthesis of norisoprenoids.



Figure 11. ¹³C-Labelling patterns of norisoprenoids: A: Expected for metabolites of ME4P derived from 1-¹³C-glucose B: Expected for metabolites of MVA derived from of 1-¹³C-glucose C: Observed for blumenol C glycoside derived from of 1-¹³C-glucose.

To clarify the contribution of either the MVA or ME4P related isoprenoid biosynthesis to the formation of carotenoid-related flavor compounds and to study the possibility of direct *de-novo* biosynthesis versus catabolic degradation of C₄₀-carotenoids, a classical experimental approach would rely on the application of radioactive, i.e. ³H- or ¹⁴C-labeled, precursor substances. However, detection of radioactive metabolites provides limited structural information and requires authentic reference substances to assure identification. Application of ²H- or ¹³C-labeled progenitors followed by isolation and applying modern two-dimensional homothorough NMR-studies and heterocorrelation experiments, such as INADEQUATE and HMQC NMR spectroscopy, unequivocally establishes structure and biosynthetic origin of norisoprenoids. Again, this approach is only suitable to identify stable end products and requires tedious isolation work to enrich minor compounds from complex samples. Consequently, an efficient analytical screening method to examine relevance of MVA or ME4P related isoprenoid biosynthesis for the formation of carotenoid-related flavor compounds should be based on HPLC-MS techniques. HPLC-MS experiments not only will allow detection of C_{13} together with their potential progenitors, norisoprenoids i.e. C15abscisic acid derivatives, C_{20} -retinoids, and C_{40} -carotenoids in one analysis as described above. From the molecular ions provided by ESI or APCI techniques one could directly extrapolate on the biochemical pathways involved: By applying for instance 1-¹³C-glucose to plants or microorganisms, the expected increase of the individual molecular mass is expected to be 8 amu for C13norisoprenoids (Figure 11B), 9 amu for C₁₅-abscisic acid derivatives and 12 amu for C20-retinoids which are synthesized via MVA. In contrast, relevance of the ME4P pathway would be demonstrated by the expected increase of the individual molecular mass of 5 amu for all C13-norisoprenoids (Figure 11A), 6 amu for C₁₅-abscisic acid derivatives, 8 amu for C₂₀-retinoids, and 16 amu for C40-carotenoids.

We already demonstrated identification of labile intermediates of the ME4P pathway by ESI-MS/MS (58). As shown in Figure 12, the electrospray process efficiently ionized phosphorylated carbohydrates such as DOX5P. Upon collisional activation, the resulting deprotonated molecules yielded characteristic product ion spectra. Again, owing to the specific *retro*-aldol type cleavage of the carbohydrate backbone one could easily differentiate isotopomers with ¹³C or ²H introduced at C-1, C-2 and C-3 from compounds labeled at C-4 and C-5.

Due to their polarity, structural variety, instability, and their noncharacteristic UV absorption, phosphorylated metabolites of the ME4P pathway are difficult to analyze by means of conventional reversed-phase HPLC. Established methods for the analysis of such small anionic compounds have been established with the help of ion exchange or ion pair chromatography. However, the high electrolyte concentration necessary for elution and the

presence of ion pairing reagents restrained the successful application of ESI for on-line coupling of these chromatographic techniques with mass spectrometry. To overcome these experimental limitations, we achieved for the first time the chromatographic separation of phosphorylated metabolites of the ME4P pathway applying a β -cyclodextrin bonded HPLC column (58). Applying acetonitrile - aqueous ammonium acetate as solvent for gradient elution, the separation shown in Figure 13 relied on the combination of hydrophilic interaction with anion exchange properties of the cyclodextrin phase in presence of ammonium ions.

As demonstrated in Figure 13, HPLC-ESI-MS/MS provides the means to identify in one analysis relevant progenitors of the ME4P pathway of isoprenoid biosynthesis together with the common metabolite IPP. Notably, any C_{13} -norisoprenoid and glycoconjugate thereof can be analyzed by that particular HPLC method, because less polar, less ionic and more hydrophobic analytes have been demonstrated to elute prior to the respective phosphate intermediates.

Summary

To gain a better understanding about intermediates involved in both the biosynthesis and the degradation of carotenoids, we discussed problems related to the ionization of carotenoids by means of the electrospray process and outlined studies focused on the identification of polar oxygenated retinoids. It is demonstrated that advanced HPLC-MS/MS experiments provide a valuable analytical basis to investigate in greater detail the relevance of the direct biosynthesis of norisoprenoid flavor substances *versus* the catabolic degradation of C₄₀-carotenoids. Finally, future experiments may clarify the contribution of the MVA and ME4P related isoprenoid biosynthesis pathways to the formation of carotenoid-related flavor compounds.

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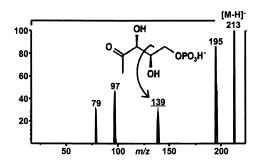


Figure 12. Product ion spectrum of DOX5P (15 eV; 2 mTorr Ar).

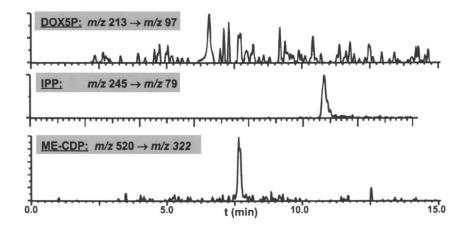


Figure 13. HPLC-ESI-MS/MS identification of ME4P related metabolites isolated from bacterial cells by specific SRM experiments (for details cf. ref. 58).

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Chapter 4

CD Spectroscopy as a Powerful Tool for the Stereochemical Assignment of Carotenoid-Derived Aroma Compounds

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The circular dichroism (CD) exciton chirality method has been extensively used in recent years for the stereochemical assignment of natural products and many other compounds. Chirality is quite often responsible for differences in the odor properties of the individual stereoisomer, especially in the case of carotenoid-derived aroma compounds. This paper gives an overview on the possibilities and limitations of the CD exciton chirality method for stereochemical identification of carotenoid-derived compounds.

Thirteen-carbon (C_{13}) norisoprenoids and related compounds are believed to be carotenoid degradation products and are common constituents in numerous plants (1). A great number of these compounds are important contributors to the overall fruit flavor and several of them (e.g. β -damascenone) also possess a considerable importance for the flavor and fragrance industry (2). Recent studies have shown that most of the volatile C_{13} -compounds identified so far are not genuine constituents of fruits but rather are derived from less or non-volatile precursors, such as polyhydroxylated norisoprenoids as well as glycosidically bound forms. Since most of these compounds are chiral, the determination of the absolute configuration became increasingly important for two reasons. First, there is a need to determine structure-odor relationships because the odor properties of stereoisomers can be considerable different, e.g. isomeric vitispiranes (3). Secondly, due to an increasing interest in the bio-oxydative

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cleavage of carotenoids, there is a need to determine the stereochemistry of the carotenoid metabolites.

The only interactions by which chiral molecules can be distinguished from their mirror image are those with a chiral environment. This environment can be either "chiral" light or other chiral molecules. The interactions with chiral, polarized light can be measured as rotation of the plane of linearly polarized light (angle of rotation) or the wavelength dependence of this angle of rotation (optical rotatory dispersion, ORD). Another measure of this interaction is the difference between the absorbance of right and left circularly polarized light as a function of wavelength (circular dichroism, CD) (4).

A lot of CD and ORD work has been done in the field of carotenoids and today most papers dealing with chiral carotenoids include CD data. In 1969 a fundamental paper was published by Bartlett et al. in which ORD spectra of carotenoids was systematically studied (5). Later, extensive CD studies of carotenoids as well as theoretical calculations have been performed by several groups (for a recent review see ref. 6). In the practical application of CD spectroscopy, the determination of the absolute configuration of carotenoids and derived compounds is still based mainly on empirical rules and on correlations with spectra of similar compounds with known stereochemistry. For this reason there is a need for nonempirical CD applications such as the CD exciton chirality method.

The CD exciton chirality method is a versatile and sensitive method for determining the absolute configuration and conformation of organic molecules containing two or more chromophores and has been widely used in the field of natural products (7,8). Hydroxyl groups are converted into various *p*-substituted benzoate groups or other chromophores, which may or may not be identical. The exciton chirality method is based on space coupling interactions of two or more chromophores in chiral substrates giving rise to bisignate circular dichroism curves. The signs and shapes of these curves are defined nonempirically by the absolute sense of twist of the interacting chromophores. Figure 1A shows the partial structure of a steroid 2,3-bis-p-substituted benzoate. Although the benzoate group can adopt various conformations, the most stable is the one depicted in which the ester bond is s-trans while the ester carbonyl and H-3 are eclipsed. Determination of the absolute configuration of the moiety shown in Figure 1A means determination of the absolute sense of chirality between the C-3-O and the C-2-O bonds. This in turn is approximated by the chirality between the two longitudinal ¹L_a electric transition moments of the benzoate chromophores, which gives rise to the main absorption band. When the chirality (orientation of the transition dipoles of the two chromophores) constitutes a clockwise turn, viewed from C-3 to C-2 or from C-2 to C-3, it is defined as positive (see Figure 1B). Such through-space coupling of the transition moments leads to a bisignate CD curve (Figure 1C) centered at the UV absorption maximum, with a positive first Cotton effect (CE) at longer wavelength and a negative second CE at shorter wavelength and vice versa. The exciton chirality method can be extended to nondegenerate systems consisting of different chromophores, as well as chromophores which already preexist in the molecule, e.g., monoene and diene moieties. Chromophores with UV maxima as far apart as 100 nm still can couple. The CD exciton chirality method has been extensively applied to cyclic molecules with rigid structures (7,8). Since the exciton chirality method depends on the conformation of the molecules, the stereochemical assignment is most straightforward and unambiguous in rigid molecules with fixed conformation. Nevertheless, the exciton chirality method has been successfully applied to acyclic 1,2- and 1,3 polyols (9), allylic and homoallylic alcohols (10), hydroxylated dienes (11), acyclic hydroxy carboxylic acids (12), α -aryl-substituted alcohols (13) as well as complex natural products (14). In the following, several examples of applications of the exciton chirality method to carotenoid derived compounds are described.

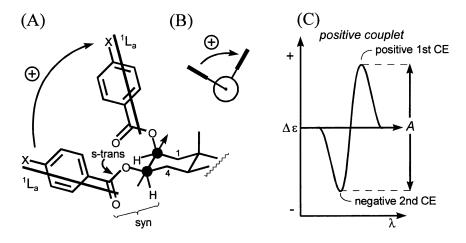


Figure 1. Principle of the circular dichroism exciton chirality method. A positive chirality (clockwise orientation of the transition dipoles of the chromophores viewed from C-3 to C-2) between the ${}^{1}L_{a}$ transition dipoles of the two benzoates gives a bisignate CD curve with a positive first Cotton effect (CE) at longer wavelength and a negative second CE at shorter wavelength (bold lines indicate transition dipoles).

Absolute Configuration of 3,4-Dihydroxy-7,8-dihydro-ß-ionol and 3,4-Dihydroxy-7,8-dihydro-ß-ionone

We have recently isolated 3,4-dihydroxy-7,8-dihydro- β -ionol 3-O- β -D-glucopyranoside 1 from gooseberry (*Ribes uva crispa* L.) leaves and 3,4-dihydroxy-7,8-dihydro- β -ionone 3-O- β -D-glucopyranoside 2 from red currant (*Ribes rubrum* L.) leaves (15,16) (Figure 2). In the course of our biomimetic studies of C₁₃ norisoprenoid precursor compounds, we showed that both compounds are precursors of important flavor compounds. Compound 1 is a precursor of isomeric vitispiranes 3 which have been found in grape juice, wine, vanilla and quince (15). Glucoside 2 is a progenitor of Riesling acetal (2,2,6,8-Tetramethyl-7,11-dioxatricyclo[6.2.1.0^{1.6}]undec-4-ene) 4, which has been identified in Riesling wine and quince brandy. Compound 2 is also a precursor of the off-flavor compound 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) 5 (16).

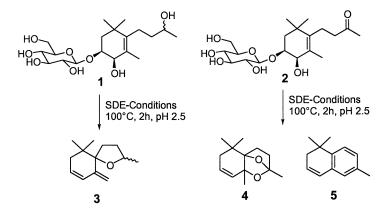


Figure 2. Structure of 3,4-dihydroxy-7,8-dihydro-β-ionol 3-O-β-Dglucopyranoside 1 and 3,4-dihydroxy-7,8-dihydro-β-ionone 3-O-β-Dglucopyranoside 2 and thermal degradation products obtained under simultaneous distillation-extraction (SDE) conditions (15,16).

In order to determine the absolute configuration, 1 and 2 were first hydrolyzed with emulsin to obtain the free aglycons 6 and 7 (Figure 3A). Treatment of 6 and 7 with *p*-dimethylaminobenzoyltriazol 8 gave the chromophoric derivatives 9 and 10 (Figure 3A). The resulting CD and UV spectra are shown in Figure 3B. The CD curve of bisbenzoate 9 shows a strong

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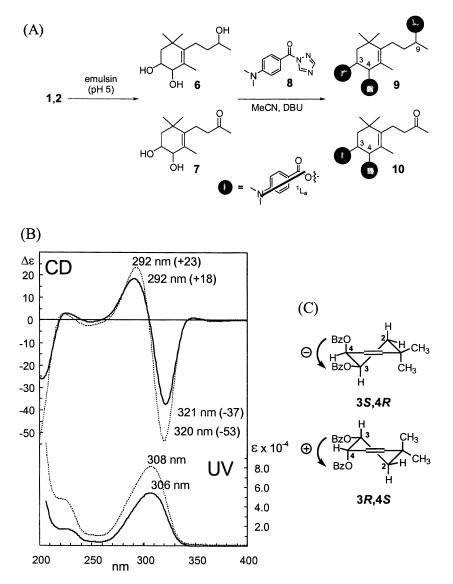


Figure 3. (A) Synthesis of chromophoric derivatives 9 and 10 (bold line indicates transition dipole) (B) CD and UV spectra of bis-(p-dimethylaminobenzoates) 9 (dashed line) and 10 (solid line) in acetonitrile, (C) possible half-chair conformations of 9 and 10 and sign of the first Cotton effect.
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negative split CD band with a negative first Cotton effect at 320 nm (-53) and a positive second CE at 292 nm (+23), amplitude A of -76. Compound 10 shows a similar negative split CD curve with extrema at 321 nm (-37) and 292 nm (+18), amplitude A = -55. The increased amplitude in the case of derivative 9 is probably due to the additional spatial coupling between the *p*-dimethylaminobenzoate in position 9 and the 3 and/or 4 benzoates, but the flexibility of the side-chain does not allow the determination of the absolute configuration of C-9. The strong negative couplings observed for 9 and 10 led to a negative chirality (counterclockwise orientation) between the ¹L_a transitions of the 3- and 4-chromophores. From NMR studies it was already known that the relative configuration of 9 and 10 is cis and that H-3 is axial and H-4 is equatorial. Furthermore computer calculations of 9 and 10 using MacroModel 5.0 and the modified Allinger MM2 force field showed that the half-chair conformation is the most preferred. These data led to either the $3S_{4}$ configuration with a negative chirality, or the 3*R*,4*S* configuration with positive chirality between the 3,4-bisbenzoates as shown in Figure 3C. Thus the obtained negative split CD curves together with NMR data established the configuration of glucosides 1 and 2 as 3S, 4R (Figure 2) (17).

Absolute Configuration of α -Ionol and 3,5,5-Trimethyl-2-cyclohexene-1-ol

Although it was not possible to obtain stereochemical information at C-9 in glucoside 9, there are other examples, e.g. α -ionol 11, where the absolute configuration can be determined using the CD exciton chirality method. At least two chromophores are required in a substrate for the application of the exciton chirality method. In the case of α -ionol, the double bond in position 7 can be used as chromophore. Since the 2-naphthoate ($\lambda_{max} = 232$ nm, $\epsilon = 54\ 000$ M⁻¹ cm⁻¹) has been employed as chromophore for exciton coupling in the past, α ionol (optical pure at C-9) was converted into the corresponding α -ionyl-2'naphthoate 13 with 2-naphthoylimidazol 12 in quantitative yield (Figure 4B). The CD and UV spectra of 13 are shown in Figure 4A. The CD spectra of chromophoric derivative 13 with a negative Cotton effect at 232 nm (-10.9) arose from exciton coupling between the transition moments of the two chromophores. In particular, the $\pi \rightarrow \pi^*$ transition of the double bond at ca. 195 nm couples with the ${}^{1}B_{b}$ transition band of the 2-naphthoate at ca. 232 nm. If the two axes of the 2-naphthoate and the double-bond chromophore possess the sense of a left-handed screw, the first Cotton effect is negative, as observed for 13 and it is not necessary to measure the second CE below ca. 200 nm. In addition to the intense $\pi \rightarrow \pi^*$ transition, the double bond has other weak transitions in the same region, and these may give rise to additional CEs at around 205 nm. Since the 2-naphthoate Cotton effect at 232 nm is isolated and not perturbed by other transitions, the sign of the first CE at 232 nm reflects the chirality in a straightforward manner (10). As previously shown for a series of acyclic allylic alcohols, the most favored conformer of the three limiting

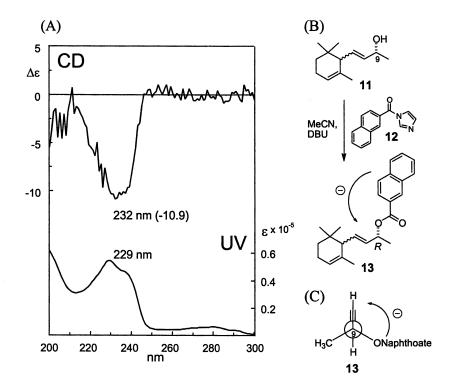


Figure 4. (A) CD and UV spectra of α-ionyl-2'-naphthoate 13 in acetonitrile,
(B) Derivatization of α-ionol 11 to the chromophoric derivative 13 using 2naphthoylimidazol 12 as reagent (C) Preferred conformation of 13.

conformers is that in which H-9 in α -ionol is eclipsed with the allylic double bond (Figure 4C) (18). These results were confirmed by computer calculations using MacroModel 5.0 and the modified Allinger MM2 force field. From this data and the obtained negative first CE at 232 nm, the 9*R*-configuration for α ionol isomer 11 was determined.

A similar example is the case of 3,5,5-trimethyl-2-cyclohexene-1-ol 14. Derivatization of 14 (optical pure enantiomers are commercially available) with benzoyltriazol 15 yielded the chromophoric benzoates (S)-16 and (R)-16 (Figure 5A). The CD and UV spectra of 16 are shown in Figure 5B. The CD spectrum of (R)-16 revealed a positive Cotton effect at 228 nm (+8.6) and the S enantiomer showed a mirror image CD with a negative CE at 227 nm (-8.4).

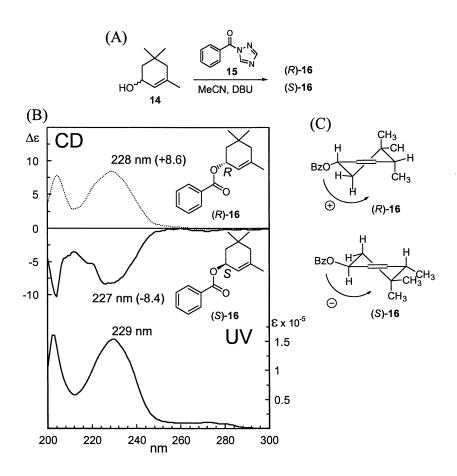


Figure 5. (A) Synthesis of chromophoric derivatives 16, (B) CD and UV spectra and (C) Half chair conformations of benzoates (R)-16 and (S)-16.

Again the CD spectra arose from the exciton coupling between the transition dipole of the benzoate chromophore and the $\pi \rightarrow \pi^*$ transition of the double bond following the same principle as discussed above for 13. In the case of cyclic allylic benzoates the application of the exciton chirality method is even easier, since it was shown that the obtained Cotton effects are independent of the conformational change of the cyclohexene ring (19). A positive CE at around 230 nm leads to R configuration and a negative CE to S configuration in a straightforward manner as shown for (S)-16 and (R)-16.

Although these examples demonstrate the usefulness of the exciton chirality method, it can not applied to 3-hydroxy-carotenoids and derived compounds like for example 3-hydroxy- β -ionol (20). The reason for this is the unfavorable alignment of the chromophoric groups; the two chromophores are almost in the same plane and the projection angle between the transition dipoles is close to 180° (exciton coupling is maximal at a projection angle of ca. 70° (8)). The amplitude of the resulting CD spectra is very weak or close to nothing and can not be used for the stereochemical assignment of 3-hydroxy-carotenoids and derived 3-hydroxy-products (20).

On-line Coupled HPLC-CD

The newest development in the field of CD spectroscopy is the *on-line* coupling with high performance liquid chromatography (HPLC-CD). Chiral separation has become one of the most important HPLC application areas in various fields, such as drug research, natural product analysis, organic and flavor chemistry. However the advances in chiral technology could not met the demands required in terms of sensitivity and selectivity. In order to overcome this problem, a new *on-line* CD detector for HPLC has been developed (21).

As one representative example, Figure 6 shows the chiral separation of the benzoates (S)-16 and (R)-16 on a Ceramospher Chiral RU-1 HPLC-column (250x4.6 mm, Shiseido, Tokyo, Japan). The UV and CD signals were monitored at 229 nm using an *on-line* CD detector (CD-1595, JASCO, Groß-Umstadt, Germany). Although the enantiomers are not baseline separated, the absolute configuration can easily be determined as S for the first eluting enantiomer and R for the second eluting enantiomer from the observed negative and positive first Cotton effect at 229 nm, respectively (Figure 6). As can be seen from this example, *on-line* HPLC-CD detection is a powerful tool which opens a new field of CD applications in the future.

Acknowledgments

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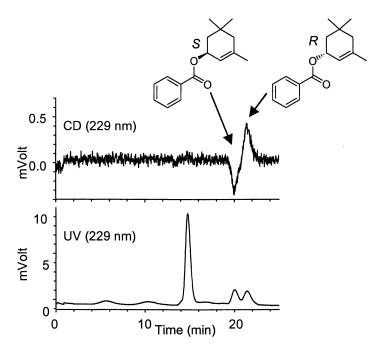


Figure 6. Chiral separation of 16 and on-line HPLC-CD detection (column: Ceramospher Chiral RU-1 (250x4.6 mm, Shiseido, Tokyo, Japan), solvent: hexane, flow: 0.4 ml/min, detector: CD-1595, JASCO, Groß-Umstadt, Germany).

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Chapter 5

Analyzing Carotenoid-Derived Aroma Compounds Using Gas Chromatography–Olfactometry

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Although the terpenes and terpenoids contained in natural products are mixtures of many chemicals only a very small number have olfactory and taste properties at their natural concentrations. Therefore, analyzing natural products for the flavor-active chemicals requires methods that are capable of distinguishing the small fraction of odor-active volatiles from the much larger number of odorless components. Gas chromatography - olfactometry (GC/O) is ideal for this purpose even though it has been underutilized for the analysis of terpenoids. In this paper we will discuss the benefits of GC/O for the analysis of aroma compounds in samples.

The study of aroma is often approached via two different methods, analytical chemistry methods and sensory science methods. Analytical chemists approach the study of aroma by measuring all volatile chemicals present whereas sensory scientists attempt to correlate sensory data with analytical data. The technique of gas chromatography - olfactometry (GC/O) brings these two groups together by providing sensory responses to chromatographically separated chemicals. The sensory scientist finds the human responses useful and convincing, while the analytical chemist finds the retention times at which the responses were made useful for chemical identification (1).

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Two methods are commonly used to analyze aroma compounds in natural products, gas chromatography – mass spectrometry (GC/MS) and gas chromatography - olfactometry (GC/O). Gas chromatography - mass spectrometry (GC/MS) is a powerful tool for the separation and characterization of chemicals whether they are odor active or not. In the analysis of aroma, GC/MS can selectively focus on the odor-active compounds once their spectral and chromatographic properties are known. However, the task of determining which compounds in a sample are odor active requires a bioassay. In other words, we must first determine "which constituent or constituents is/are contributing to the characteristic sensory properties of the food product being investigated" (2). GC/O is a bioassay that reveals odorants in terms of their pattern of smell-activity thus eliminating odorless compounds from consideration.

It has been shown that the human detector is much more sensitive than the chemical detector. One reflection of this sensitivity is seen in the detection of ßdamascenone in natural products. B-Damascenone has been found to be one of the most potent odorants in many food products. Its characteristic floral odor contributes to the odor character of apples (3), various grape varieties, beer, coffee oil, buchu leaf oil, Satsuma mandarin (4), rhambutan (5), lychee (6), textured soy protein (7), soymilk (8), tobacco, tea, raspberry oil (9), and cane molasses (10). However, β -damascenone is often not detected by GC/MS analysis. This is due to the low odor threshold of β -damascenone, 2 - 20 pg/g in water (11), which means that trace amounts of β -damascenone are often detectable by smell but not by chemical detectors. Other compounds for which this is often the case are β -ionone, 2-acetyl-1-pyrroline, geosmin, and 2-secbutyl-3-methoxypyrazine. Figure 1 shows a Venn diagram that is representative of the current situation in most aroma research. There is a degree of overlap between the compounds detectable by GC/O and GC/MS analysis. However, GC/MS analysis alone will not detect some of the odor active compounds in a natural product, often missing the most odor potent chemicals in a sample. The percentage of odorants missed varies, however, it can be stated that just using GC/MS over emphasizes the importance of some compounds well under emphasizing the importance of others.

It is important to note that GC/MS fails to indicate which compounds are odor active in a sample as well as how odor potent the compounds are. This is an important issue because GC/MS analysis often detects "odorants" although they may not be above their odor. For example Ong et al. identified 51 Downloaded by UNIV OF MICHIGAN on July 2, 2011 | http://pubs.acs.org Publication Date: November 21, 2001 | doi: 10.1021/bk-2002-0802.ch005

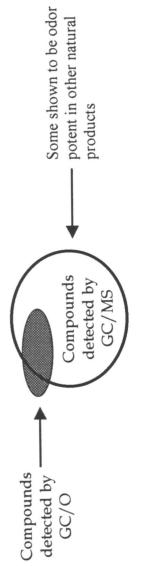


Figure 1. Venn diagram showing the situation in aroma research.

compounds via GC/MS analysis that did not possess odor activity in rhambutan fruit (5) even though they have been shown to have odor activity in other natural products. For example, hexanal has been shown to be a lipid oxidation product and one of the most odor potent compounds in soy milk as well as one of the major contributors of the beany odor of soymilk (8). Therefore, the use of GC/MS to analyze the odorants in a sample is seldom representative of the odor profile of that sample although it is a measure of the volatile components of that sample.

Table 1 below shows two specific examples that incorporated both GC/MS and GC/O to analyze the aroma active compounds in two natural products, bell pepper and rhambutan fruit. Seventy-four total compounds were identified in bell pepper, of these 14% were detected only by GC/O analysis. If GC/O analysis had not been used one third of the odor active compounds in the bell pepper sample would have not been detected. In the rhambutan study 112 total compounds were detected by the combination of GC/O and GC/MS analyses, GC/O helped to identify 11% of the total compounds detected. This accounts for 20% of the odor active compounds in rhambutan fruit.

Table 1. Number of compounds detected by two analytical methods of aroma analysis.

^{*a*}Luning et al., 1994 (*14*). ^{*b*}Ong et al., 1998 (*5*,*6*).

When analyzing carotenoid derived aroma compounds for odor activity, a bioassay must be performed to determine if the compounds possess odor in the product they are extracted from. Table 2 lists some common carotenoid derived aroma compounds and their corresponding odor descriptor. These compounds are often found to be one of the most odor potent compounds in a sample. However, they are also found in products where they do not contribute to the odor. For example, Ong et al. (5,6) found β -ionone present in rhambutan fruit using GC/MS analysis. However β -ionone was not found to be odor active in the fruit because β -ionone was not above its odor threshold. Another common carotenoid derived aroma compound, β -damascenone, however, was found to be the most potent odorant in rhambutan.

	Compound	Odor descriptor
	α-Damascone	fruity, woody, violet
	ß-Damascone	violet
	β-Damascenone	floral
	α-Ionone	woody
	β-Ionone	nori-like
		n of the GC/FID chroma
bectrum	value (USV) chromat	togram of rhambutan fi

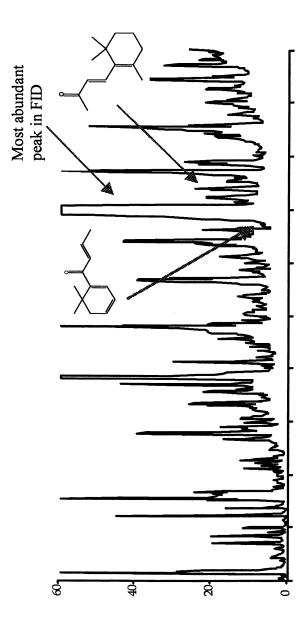
Table 2. Odor descriptors of common carotenoid-derived aroma compounds.

e GC/FID chromatogram and an odor sp of rhambutan fruit. Plotting odor spectrum values versus retention index produces an OSV chromatogram. Odor spectrum values are normalized flavor dilution values or Charm values and modeled on Steven's law,

 $\Psi = k\Phi^n$

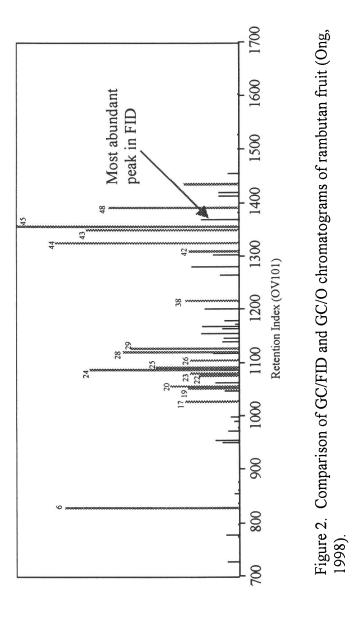
where Ψ is equal to the perceived intensity of a stimulant, k is a constant, Φ equals the stimulus level, and n is Steven's exponent. Steven's law exponent for odorants range between 0.3 and 0.8 (12,13). An OSV chromatogram is representative of the pattern of odorants in the sample and is independent of concentration. As shown in Figure 2 many of the most odor-active compounds in rhambutan, OSV above 30% indicated by gray in the OSV chromatogram, were either not detectable or were minor peaks in the GC/FID chromatogram. The most potent odorants in rhambutan fruit, listed by peak number, were found to be 6- ethyl-2-methyl butyrate, 17- furaneol, 19- m-cresol, 20- guaiacol, 22nonanal, 23- 2-phenylethanol, 24- unknown 1 (sweaty odor), 25- heptanoic acid, 26- maltol, 28- (Z)-2-nonenal, 29- (E)-2-nonenal, 38- phenylacetic acid, 42hydrocinnamic acid, 43- (E)-4,5-epoxy-(E)-2-decenal, 44- vanillin, 45- β damascenone, 48- cinnamic acid, 51- 8-decalactone. Also, many of the major volatiles in rhambutan contribute little to the GC/O chromatogram. The most abundant peak in the GC/O chromatogram, β-damascenone, was the only carotenoid derived compound in rambutan above its odor threshold and it was not detectable in the GC/FID chromatogram.

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In Carotenoid-Derived Aroma Compounds; Winterhalter, P., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

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These examples show that it is important to use GC/O if you are interested in the odor active chemicals in a sample. If only GC/MS is used to study the aroma of a sample or extract the odor importance of many chemicals will be over emphasized and many of the most important odorants will be under emphasized or not detected. It is also important to note that of the hundreds of terpenoid compounds present in a sample only a small subset possess odor in natural products.

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Chapter 6

Carotenoid Cleavage Enzymes in Animals and Plants

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Carotenoid cleavage enzymes have been investigated for more than 50 years. Nevertheless, progress in this area has been slow and a complete understanding of the reaction mechanisms of carotenoid biodegradation has not been achieved at the present time. In this study, the enzymatic cleavage of carotenoids in several insect species (Calliphora, Ornithoptera, and Locusta) is examined. The enzymatic degradation of carotenoids in plants is also elucidated. Quince (Cydonia oblonga) and star fruit (Averrhoa carambola) were examined in detail, since both fruit are known to contain major amounts of carotenoid derived C_{13} norisoprenoid aroma compounds. Our data demonstrates that carotenoid cleavage enzymes purified from animal tissues are similar to each other, whereas carotenoid cleavage enzymes obtained from fruit are clearly different and readily distinguished.

The first report concerning carotenoid cleavage enzymes was published 60 years ago (1). However, knowledge about kinetic properties, protein structure and reaction mechanisms of carotenases has progressed slowly in the ensuing

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years. Many questions concerning carotenases are still the subject of an ongoing debate. The enzymatic reaction mechanism of these enzymes, for example, is still debated (2,3) and the elucidation of the major pathways of carotenoid biodegradation remains unresolved. Furthermore, little is known about carotenoid cleavage in animal systems other than vertebrates (4). The knowledge of enzymatic catabolism of carotenoids in plants is also quite limited. Since the flavor of several plants (flowers and fruit) is heavily dependent on carotenoid derived C₁₃ norisoprenoids, we have attempted to contribute to the understanding of carotenoid metabolism in plants. To achieve this goal, two species containing high amounts of norisoprenoid constituents: quince (*Cydonia oblonga*) and star fruit (*Averrhoa carambola*) were carefully analyzed (5). The data obtained from the fruit-derived carotenases will be compared with those from well-known mammalian systems and some insect carotenases. These latter carotenases were also recently investigated by our group (6).

Methods

Protein Isolation

Carotenoid cleavage enzymes were isolated from fruit and animal tissues using different schemes. Commercially available star fruit and quince fruit were stored in the lab for one week until fully ripe. It is important to note, that in the case of quince, the fruit had to be kept in a cold room at $3-5^{\circ}$ C for at least two weeks to induce fruit ripening. After peeling, the fruit skin tissues (peel) were homogenized in sample buffer (125 mmol/l KCl, 5 mmol/l MgCl, 50 mmol/l Tris, pH 6.8) using a Teflon homogenizer (120 s, 1500 rpm). The resulting homogenates were centrifuged at 2000 g for 10 min and the supernatant was again centrifuged (10,000 g, 120 min). The supernatant of the second centrifugation was subjected to an acetone precipitation (85 %, 2 h). The resulting precipitate was rediluted in sample buffer and transferred to a preparative isoelectric focusing unit (Bio-Rad Rotofor, pH 3-10). In order to remove the ampholytes needed for the isoelectric focusing process, all protein fractions were ultrafiltrated at 50 kD. The workup was carried out at 4°C.

Animal enzymes were isolated in a similar manner from the intestines of blowflies (*Calliphora erythrocephala*, mut. chalky), locusts (*Locusta migratoria*) and wistar strain rats. However, in the case of birdwing butterflies (*Ornithoptera priamus poseidon*), eyes were used for the preparation of carotenoid cleavage enzymes. The carefully flushed intestines as well as the freshly cut eyecups were homogenized as described above for fruit tissues. pH values of the buffered samples were 7.4 (rats) and 7.0 (insects), respectively. Homogenates were subsequently centrifuged twice (120 s, 1500 g and 120 min, 50000 g). The resulting supernatant was ultrafiltrated at 300 kD and the filtrate subjected to the isoelectric focusing. After this step, the enzyme fractions obtained from plant and animal sources were treated equally.

Sample Preparation

To facilitate the accessibility of the hydrophobic carotenoid substrates to the water soluble cytosolic enzyme fractions, β -carotene was added to the samples in a micellar solution of Tween 40 and water. The β -carotene solution was prepared by diluting 1.0 mg of β -carotene in a solution of acetone (5.0 ml) and Tween 40 (1.0 g), followed by the complete removal of acetone using a rotary evaporator. The substrate stock solution was obtained by diluting the solution of β -carotene in Tween 40 up to 10.0 ml with water (Milli Q).

Enzymatic experiments using isolated proteins were carried out at room temperature in a micro cuvette. Total sample volume was 375 μ l. Except for the Lineweaver Burke experiments, initial substrate (β -carotene) concentration was 2.48 μ mol/l. The reaction mixture consisted of sample buffer, an appropriate amount of β -carotene stock solution as well as the isolated enzymes. No additional substances were added. Oxygen, needed as a second substrate for the oxidative cleavage of carotenoids, was provided by the air saturated sample buffer solutions. All kinetics of the carotenoid cleavage reactions were monitored continuously with an UV/VIS spectrometer at 505 nm. At this wavelength, it was possible to monitor the degradation of β -carotene uninfluenced from spectral absorptions of the resulting cleavage products.

Electrophoresis Conditions

The SDS PAA electrophoreses experiments employed commercial gels (PSGEL NPG 520, 8%-20% PAA, Atto Corp., Japan) to elucidate the molecular weight of the fruit carotenases. The gels were stained using a commercially available kit (Silver Stain Kit, WAKO Corp. Japan). Gels were run under constant voltage conditions (30 V) at room temperature.

Results

Animal Carotenases

Carotenoid cleavage activity was detected in both animal and fruit tissues. As might be expected from the results of previous studies (7-9), carotenoid cleavage enzymes derived from animals were found in intestinal extracts from rats, blowflies and locusts. In the case of birdwing butterflies no carotenoid cleavage activity was detectable in their intestinal extracts (10), but carotenase activity could be isolated from eyecups.

Some of the kinetic data is presented in the following figures in order to compare the carotenases of animal origin with those isolated from plants. Figure 1 shows the rate of β -carotene degradation using three carotenases. In each case the reaction can be fitted to an exponential decay function and thus can be considered a first order reaction.

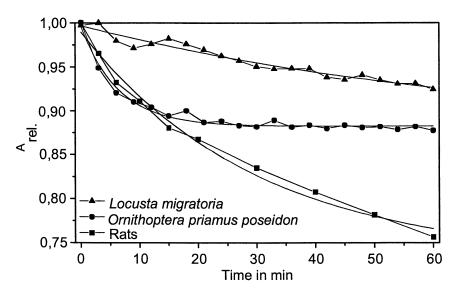


Figure 1. Time courses of β -carotene bleaching using different enzyme preparations from insects and rats (for detail cf. text).

The exponential equations describing the carotenoid cleavage are:

 $y = 0.86 + 0.13 e^{-(x/77.5)} (Locusta)$ $y = 0.82 + 0.11 e^{-(x/6.3)} (Ornithoptera)$ $y = 0.73 + 0.26 e^{-(x/30.1)} (Rats)$

 $y = 0.95 + 0.04 e^{-(x/9.4)} (Calliphora^*)$

(* not shown in Figure 1)

The time constants of these reactions are: 77.5 min, 6.4 min, 30.1 min and 9.4 min for *Locusta*, *Ornithoptera*, rats and *Calliphora*, respectively. Results for the blowfly *Calliphora erythrocephala* are not shown in Figure 1, because these experiments were done using a different time scale.

Since all the reactions have been shown to be first order, they can be analyzed by the Michaelis-Menten kinetics. The results of these experiments are presented in Table I and Figure 2.

	Rats	Calliphora	Ornithoptera	Locusta
Molecular Weight (kD)	200-250 ¹	159	159	-
Isoelectric Point (pH)	7-8	10.5	11	10.5
Opt. Temperature (°C)	35	40	40	42
Opt. pH	7	7	-	6.8
$K_{M} (\mu mol \cdot l^{-1})$	-	3.0	-	-
$V_{max}(\mu mol \cdot l^{-1} \cdot min^{-1} \cdot mg_{Prot}^{-1})$	-	1.1•10 ⁻³	-	-
Time Constant (min)	30.1	9.4	6.4	77.5
Active Tissues	Intestine; Liver	Intestine	Eyes	Intestine

Table I. Kinetic and Physical Key Data of Animal Carotenases

¹Data taken from (7)

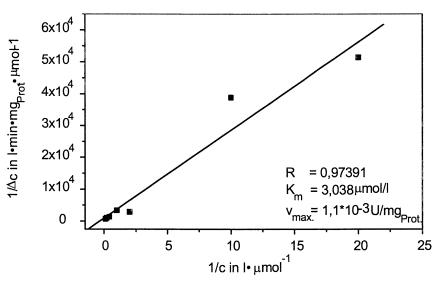


Figure 2. Lineweaver Burke plot of blowfly carotenoid cleavage enzyme.

For the blowfly system, the Michaelis constant $(K_M = 3.0 \ \mu mol^{-1})$ as well as the maximum velocity $(v_{max} = 1.1 \cdot 10^{-3} \ \mu mol^{-1} \cdot min^{-1} \cdot mg_{Prot.}^{-1})$ was determined. Since we used rats only as a reference system to our insects - and due to the very limited butterfly and cricket material available - Figure 2 shows only data of blowfly enzymes. However, in Table I additional literature data for rat enzymes are given.

Table I also shows additional kinetic data for other animal derived carotenoid cleavage enzymes. The optimal temperature is 35°C for rat carotenase enzymes, 40°C for *Ornithoptera* and *Calliphora* enzymes and 42°C for *Locusta* enzymes (Table I). Optimal pH is also given for rats (pH = 7), locusts (pH = 6.8) and blowflies (pH = 7) (11).

In addition to the kinetic data, some physical properties of animal carotenases were also determined. The molecular weight of mammalian carotenoid cleavage enzymes is known to be around 200 kD (7). Similar data for insect carotenases have never been reported. We determined the molecular weight of carotenases from *Calliphora* and *Ornithoptera* to be 159 kD (Table I). Furthermore, the carotenases' isoelectric points were also determined. For rats, it is at pH 7-8, whereas for insects it is clearly in the alkaline region (*Calliphora* and *Ornithoptera*: pH = 10.5; *Locusta*: pH = 11) (6).

Fruit Carotenases

For the isolation of plant-derived carotenoid cleavage enzymes we used skins from quince (*Cydonia oblonga*) and star fruit (*Averrhoa carambola*). The rate of β -carotene bleaching using plant-derived enzymes is shown in Figure 3.

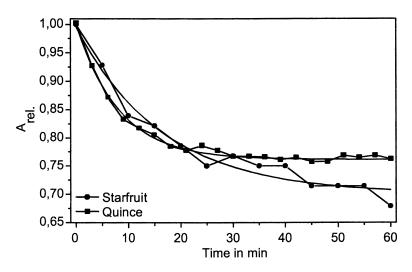


Figure 3. Time course of β -carotene bleaching using plant-derived carotenases.

Again, the data could be fit to an exponential first order decay and therefore the carotenoid cleavage reaction in star fruit and quince can be assumed to be a first order reaction, similar to animal carotenases (cf. Fig. 1). The exponential functions are:

> y = $0.69 + 0.29 e^{-(x/16.6)}$ (star fruit) y = $0.99 e^{-(x/8.2)}$ (quince)

The calculated time constants of the carotenases are 16.6 min for star fruit and 8.2 min for quince (Figure 3). The Michaelis-Menten approach could be applied to fruit carotenases in the same way as to animal carotenases in order to determine key kinetic data, i.e. the Michaelis constant and the maximum velocity. The Michaelis constant for quince enzymes is $K_M = 11.0 \ \mu mol^{-1}$ (Table II), whereas for star fruit it is $K_M = 3.6 \ \mu mol^{-1}$ (Table II, Figure 4). The maximum velocity for quince and star fruit is $v_{max} = 0.083 \ \mu mol^{-1} \ min^{-1} \ mg_{Prot.}^{-1}$, respectively. For quince carotenases the optimal temperature is above 50°C, for star fruit 45°C. The optimal pH could only be determined for star fruit (pH = 8.5), because the activity of quince enzymes showed no differences due to the pH of the reaction mixture (Table II).

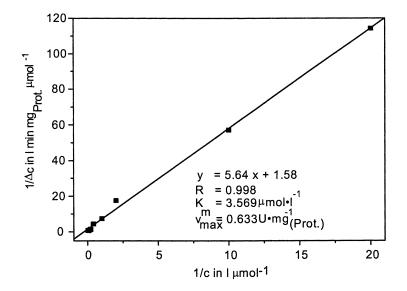


Figure 4. Lineweaver Burke plot of carotenoid cleavage enzymes from star fruit.

Table II. Kinetic and Physic	al Properties	of Fruit C	Carotenases
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	Quince	Starfruit
Molecular Weight (kD)	20-30	80-90
Isoelectric Point (pH)	2.8	3.5
Opt. Temperature (°C)	>50	45
Opt. pH	-	8.5
K_{M} (µmol•l ⁻¹)	11.0	3.6
$V_{max}(\mu mol \cdot l^{-1} \cdot min^{-1} \cdot mg_{Prot}^{-1})$	0.083	0.63
Time Constant (min)	8.17	16.6
Active Tissues	Fruit Skin	Fruit Skin

The molecular weight and the isoelectric point of the fruit carotenases were also determined (Table II). For quince, the isoelectric point was found to be at pH = 2.8, for star fruit it was pH = 3.5. The molecular weight (size), also given in Table II, was in the range of 20-30 kD for quince and 80-90 kD for star fruit. We could isolate these carotenoid cleavage enzymes to a state, where 4 (quince) and two (star fruit) protein bands were left, when the active samples were analyzed by SDS PAA electrophoresis. The SDS PAGEs of the quince proteins did not show differences between reducing and non reducing conditions.

Finally, Table III shows a comparison between the kinetic and physical properties of animal and plant (fruit) carotenases currently known. Of the seven enzyme properties measured, five (molecular weight, IEP, opt. (optimal) pH, K_M and v_{max}) are similar within the group of animal and plant proteins, but clearly different between these groups. The two remaining ones, i.e. optimal temperature and time constant, show almost no differences between plants and animals (optimal temperatures) or are widely overlapping (time constants).

	Animals	Plants	
Molecular Weight (kD)	160-250	20-90	Different
Isoelectric Point (pH)	7-11	2.8-3.5	Different
Opt. Temperature (°C)	35-42	45->50	Different
Opt. PH	6.5-7.0	8.5	Different
K_{M} (µmol•l ⁻¹)	3.0	11.0-3.6	Similar
V _{max.} (l•min•mg _{Prot.} •µmol ⁻¹)	1.1 x 10 ⁻³	8.3 x 10 ⁻² - 0.63	Different
Time Constant (min)	6-78	8-17	Similar
Active Tissues	-	-	-

Table III. Comparison of Animal and Fruit Carotenases

Discussion

Previous Studies

Carotenoid cleavage enzymes have been investigated for many decades (1,2). However, almost all these studies focused on the analysis of carotenoid cleavage in mammals. A few studies also considered carotene metabolism in birds (12,13). Since the majority of these studies are based on a medical interest,

little is known about carotenases from non mammalian sources. Almost nothing is known about carotenases from non vertebrate origins.

Most prior studies have focused on the elucidation of carotenoid cleavage reaction products. Therefore the current knowledge of carotenase reaction mechanisms is still limited. In fact, it is almost unchanged since 1960 when Glover (2) proposed that carotenoids might be enzymatically cleaved by a dioxygenase either at the central double bond or at the 8,8' double bond. Since that time, various studies supported either one or the other hypothesis, in terms of the reaction products obtained (8,14,15). The only modification to this scheme was introduced around 25 years ago with the proposal of random cleavage instead of central or excentric cleavage (12). This random cleavage theory proposed that all double bonds of the carotenoid can be cleaved "randomly" by carotenases (16).

All prior studies employed enzymatically active protein fractions isolated from the tissues used, mostly liver and intestinal mucosa (17-20). Up to this time it has not been possible to isolate any of these carotenases to a single protein state. Consequently, only limited data on the physical properties of animal carotenases have been reported. Our current knowledge is limited primarily to the fact that carotenases are cytosolic enzymes (3,16) and proteins have an approximate size of 200 kD (7).

Only recently have a few other carotenoids such as lutein, lycopene or zeaxanthin been used as substrates for carotenoid cleavage enzymes. Most of these studies have shown that the action of these carotenases is inhibited in the presence of carotenoids other than β -carotene (21,22).

Insect and Fruit Carotenases

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The primary goals of our insect carotenase investigations were to find out if there are different carotenases other than those known in vertebrate systems and whether insect systems are similar or different from vertebrate systems. We were also interested in searching for plant derived carotenoid cleavage enzymes, because it has not been possible to find anabolic pathways leading to C_{13} compounds which are important contributors to the flavor of fruits (24).

Insect Carotenases

The carotenases we found in insects proved to be more or less similar to vertebrate enzymes. The molecular weight (size) was slightly lower than expected (Table I), whereas optimal temperature, pH, and Michaelis constant were similar to vertebrate systems (Table I). The time constants could not be separated significantly from our reference system (rats) because of the high variation within the investigated insect species (Table I). Nevertheless, some of the time constants are rather similar to those found in fruit enzymes (Table II). On the other hand, the isoelectric point and the maximum velocity of insect carotenases are clearly different from vertebrate enzymes. In the case of isoelectric points, those of insect enzymes proved to be shifted far to the alkali region (Table I). Under our reaction conditions, the maximum velocity of blowfly carotenases was three orders of magnitude higher than that of rat enzymes (Table I).

The reaction mechanism of insect carotenases, however, seems to be exclusively of the "excentric cleavage" type, since no retinal or shorter apocarotenoids like β -apo-10'carotenal could be found as reaction products (4). Despite the fact that insect carotenoid cleavage enzymes are exclusively located in the intestine in all but one species (Table I), the absence of retinal separates these proteins from the vertebrate enzymes at least on a functional level. The exception from the "intestinal carotenases" is the only lepidopteran species tested for the presence of carotenoid cleavage activity could only be found in eye tissues (Table I). The intestine of Ornithoptera did not show any carotenase activity (10). So far, this difference could not be attributed to any ecological or evolutionary reasons, but further investigations are currently in progress.

Even though the knowledge about carotenoid cleavage enzymes derived from insect tissues is still limited, one of these enzymes (from *Calliphora erythrocephala*) is the only one so far, which has been purified to the single protein level (24). Although the purification has only been done on an analytical scale, it facilitates comparative approaches to the elucidation of carotenoid cleavage both in plants and other animal sources.

Plant Carotenases

As previously noted, our interest in plant carotenases was based on the fact that fruit contain a series of carotenoid derived C_{13} norisoprenoids as key flavor compounds (5). Among various species, the fruit of *Cydonia oblonga* (quince) and *Averrhoa carambola* (star fruit) are well known sources for these C_{13} norisoprenoids (23,25). Therefore, we focused on these fruits in order to isolate for the first time, carotenoid cleavage enzymes from plant sources. In both cases, we found that only the skin tissue of the fruit contained sufficient amounts of carotenoid cleavage enzymes (Table II).

The kinetic properties of the two fruit carotenases showed some similarities but also differences. This is not surprising for enzymes isolated from systematically very distant species. Quince (*Cydonia*, Rosaceae) and star fruit (*Averrhoa*, Oxalidaceae) are only related on the level of the sub-class Rosidae (26). In detail, the values for time constants, optimal temperatures and maximum velocities can be regarded as similar. However, the Michaelis constants and the optimal pH of the carotenoid cleavage enzymes from these fruit show clear differences (Table II).

With regard to the physical properties of fruit carotenases, until now we could only determine the molecular weight and the isoelectric point. The isoelectric point in both cases is strongly acidic (Table II). However, the molecular weights are clearly different (Table II), even if purification of the enzymes has only reached a level of four (quince) and two (starfruit) remaining proteins.

Comparing Animals and Plants

Since plant carotenases have been isolated for the first time, it is of special interest to compare our data with carotenoid cleavage enzymes derived from animal sources. Despite the obvious heterogeneity, mainly of animal carotenases, plant and animal derived carotenoid cleavage enzymes are different in all but two properties studied (Table III). Molecular weight, isoelectric point, optimum temperature and pH, as well as the maximum velocities all show a clear gap between animals and plants. In some cases these differences are as large as several orders of magnitude (pH and v_{max}, cf. Table III). However, Michaelis and time constants of the carotenoid cleavage reactions are not qualified as group separating parameters. The values of the time constants overlap completely, whereas the Michaelis constants of plant carotenases are only similar to the lower Michaelis constants obtained for animal carotenases (cf. Table III), mainly due to their much higher variability. Only the lower end of animal K_M values overlaps with plant enzymes. Reviewing what is known about carotenases from animals and plants, it seems to be obvious that carotenases form two clearly distinct groups: animal and plant carotenases.

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Identification of β-Damascenone Progenitors and Their Biogenesis in Rose Flowers (Rosa damascena Mill.)

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> Flowers of Rosa damascena Mill. were found to contain enzymatic activity that potentially oxidized or cleaved carotenoids to yield C₁₃-norisoprenoids. The flowers also potential to generate C_{13} -norisoprenoids showed the enzymatically from endogenous carotenoids, such as β carotene, neoxanthin, and zeaxanthin, which were also identified in the flowers. We could detect several progenitors of β -damascenone, the most intense C₁₃-norisoprenoid volatile compound among the constituents of rose essential oil. One of the progenitors was isolated and its chemical structure elucidated as 9-O-β-D-glucopyranosyl-3-hydroxy-7,8-didehydro-β-ionol.

The flowers of *Rosa damascena* Mill. are utilized for the production of an essential oil, in which several volatile C_{13} -norisoprenoids are present. One of

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the key flavor compounds is β -damascenone (1), possessing an extremely low sensory threshold (2 pg/g of water) (1). Ketone 1 was first identified in Bulgarian rose oil in 1970 (2). Since then, this compound was also identified in various types of plant tissues and beverages (3-18) as shown in Figure 1. Aroma compound 1 itself is neither produced nor released from the flowers of Bulgarian rose (*R. damascena* Mill.). Rose essential oil, however, contains this compound as a key constituent. It has been suggested that ketone 1 is produced from certain progenitors during the steam distillation of rose flowers.

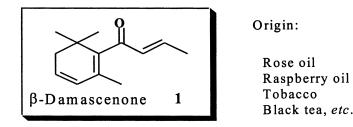


Figure 1. β -Damascenone (1) and its origin.

Not only ketone 1 but also many other C_{13} -norisoprenoids are suggested to be produced from reactive progenitors, such as, e.g., glycosidic or polyhydroxylated compounds upon heat treatment and/or acidic hydrolysis (19-27). C_{13} -norisoprenoids are considered to be derived from carotenoids (28-34). Rosa damascena Mill. produces volatile C_{13} -norisoprenoids in smaller amounts compared to tea roses with yellowish color (35). This is obviously due to the differences in the carotenoid content. In tea roses carotenoids are present in considerably higher amounts. As shown in Figure 2, β -ionone may be directly produced upon oxidative cleavage of β -carotene between positon C-9 and C-10. Grasshopper ketone, 3-hydroxy- β -ionone, and 3-hydroxy-5,6-epoxy- β -ionone are similarly produced from neoxanthin (36,37), lutein, and violaxanthin, respectively. These primary degradation products (cf. Figure 2) of enzymatic cleavage are further transformed in plant tissues. Through enzymatic reduction of, e.g., grasshopper ketone an allenic triol **2a** (cf. Figure 5) is obtained, which is a known progenitor of rose ketone **1**.

This paper describes (i) identification of carotenoids being putatively involved in the formation of C_{13} -norisoprenoid compounds, (ii) screening of carotenoid cleavage enzymes, and (iii) characterization of progenitors of ketone 1 in flowers of *R. damascena* Mill.

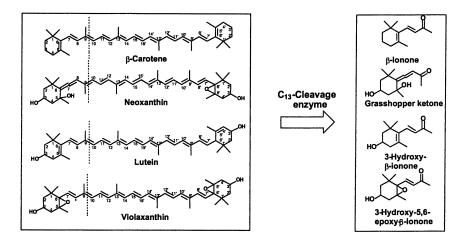


Figure 2. Proposed pathway for carotenoid degradation.

Experimental

Identification of Carotenoids in *R. damascena* Mill. The flowers of *R. damascena* at the opening stage were homogenized in liquid nitrogen to give a fine powder. A portion (10 g) of the powder was extracted with 100 mL of MeOH under ice cooling for 4 hr. To the filtrate of the extract were added hexane (100 mL), diethyl ether (100 mL), and water (20 mL). Then the phases were partitioned to give an upper layer, which was washed with the mixture of 100 mL of MeOH and 20 mL of water. The upper layer was saponified in the usual manner with 30% KOH in MeOH for 13 hr at 4°C. Carotenoids were recovered in a hexane-diethyl ether fraction to give dried materials (26.9 mg) after evaporation *in vacuo*. The carotenoids were analyzed with HPLC (Jasco Model MD-1510, Japan) equipped with a diode-array detector and a column from Shiseido Capcel Pak C18 UG 120A-5, 4.6 x 250 mm. The eluent and the gradient conditions were identical to the method developed by Deli *et al.* (42), except for the column oven temperature (40°C) and the flow rate (1.0 mL/min) of the solvent.

Detection of Carotenoid-cleavage Enzymes in Flowers of R. damascena. Flowers at opening stage were grounded in liquid nitrogen to give a fine powder (2.5 g), which was extracted twice with 10 mL of tris-HCl buffer (0.1 M, pH 8.0) containing 1 mM dithiothreitol for 5 min under ice cooling. After centrifugation (1,400 g, 10 min), the supernatant was recentrifuged under the same conditions as mentioned above to give a supernatant, which was used as a crude enzyme extract. β -Carotene and neoxanthin were employed as substrates as described by Fleischmann *et al.* (38). The carotenoid cleavage enzyme activity was estimated by monitoring the absorption at 500 nm.

Isolation and Characterization of β-Damascenone Progenitors from the Flowers of R. damascena Mill. The frozen flowers at full bloom stage (10 kg) were extracted with 70% aqueous MeOH under ice cooling and the extract was subjected to a column of Amberlite XAD-2, which was developed with MeOH to give the progenitor fraction. The fraction was acetylated (pyridineacetic anhydride) and purified by flash chromatography (hexane-EtOAc) on silica gel to yield 8 fractions. For the detection of β -damascenone progenitors, a portion of the fractions was deacetylated by treatment with NaOMe, and heated at 90°C for 2 hr at pH 2 in a sealed tube. Volatile compounds formed were extracted under neutral conditions by the azeotropic mixture of pentane- CH_2Cl_2 (2:1) and analyzed by GC-MS. For GC-MS JEOL JMS-DX-302 with a MS data system JEOL JMA-DA 5000 and a Hewlett Packard 5890 GC (column PEG-20M (0.25 mm x 50 m); linear temperature gradient from 60°C to 220°C (3°C/min); injector temperature: 150°C; carrier gas: He at 1 mL/min) was used. Fractions A-3 and A-5 were further purified by column chromatography on silica gel (hexane-TBME-gradient) followed by HPLC (column: ODS-AM, YMC, Japan, Fluofix-120N, NEOS, Japan; solvent: MeCN-water) to give β damascenone progenitor fractions, A1-B4 (compound 4, 1.1 mg), A3-B5+6-C7-D4+5, A5-E4-F10-G5+6, and A5-E4-F14+15.

Synthesis and Identification of Allenic triol (2a) and its 3-Oglucopyranoside (2b). Allenic triol (2a) was synthesized according to the method published by Mori (45). (6R)-2,2,6-Trimethyl-1,4-cyclohexadione and 3-butyn-2-ol were used as starting materials. The epoxidation of acetylenic diol with MCPBA gave a mixture of α - and β -epoxides. Desired α -epoxide was used for the further reactions. Triol 2a was obtained as an optically pure compound. Oxidation of 2a gave grasshopper ketone.

3-O-Glucopyranoside of allenic triol (2b) was synthesized in 7 steps (details will be reported elsewhere) starting from 3-hydroxy-7,8-didehydro- β -ionone. An allenic proton (H-8) appeared as two sets of doublet signal at δ 5.45 and 5.47 (1H, d, J = 5.4 Hz), indicating that 2b was obtained as a diastereomeric mixture. Compound 2b gave a single sharp peak by HPLC analyses under the following conditions; column: YMC-ODS-AM5, 4.6 x 250 mm (Yamamura Chemical Co. Ltd, Kyoto, Japan); solvent: 5% MeCN (0-10 min), then increased the concentration of MeCN to 30% in 30 min at a flow rate of 1.0 mL/min at 40 C; detector: photodiode array (Jasco MD-1510, Japan).

Results and Discussion

Identification of Carotenoids in Flowers of R. damascena Mill.

From the flowers of *R. damascena* a carotenoid fraction was obtained. As shown in Figure 3, at least ten individual carotenoids were detected including β carotene ($t_R = 65.3 \text{ min}, \lambda_{max} 453, 475 \text{ nm}$), zeaxanthin ($t_R = 34.8 \text{ min}, \lambda_{max} 445$, 475 nm) and neoxanthin ($t_R = 15.5 \text{ min}, \lambda_{max} 437, 467 \text{ nm}$) in the flowers of *R. damascena* at opening stage. The content of each of the above mentioned carotenoids was 13, 8, and 0.2 mg/kg of fresh flowers, respectively, based on quantitative analyses employing authentic specimens. As already reviewed by Eugster and Märki-Fischer (32), these carotenoids have been previously identified in rose flowers. The authors also reported the presence of several carotenoid-derived degradation compounds in rose flowers. Thus, we expected the presence of carotenoid cleavage enzymes in rose flowers being capable of cleaving the presumed parent carotenoid neoxanthin. As a result of this cleavage, formation of reactive progenitors of β -damascenone (1) in flowers of *R. damascena* is likely to occur.

Detection of Carotenoid Cleavage Enzymes in the Flowers of *R. damascena* Mill.

Figure 4 shows the time course of β -carotene cleavage by the crude enzyme prepared from the flowers at the opening stage.

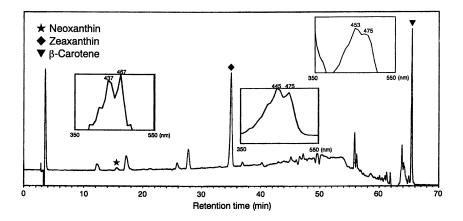


Figure 3. HPLC analytical data and UV spectra of carotenoids obtained from flowers of R. damascena Mill.

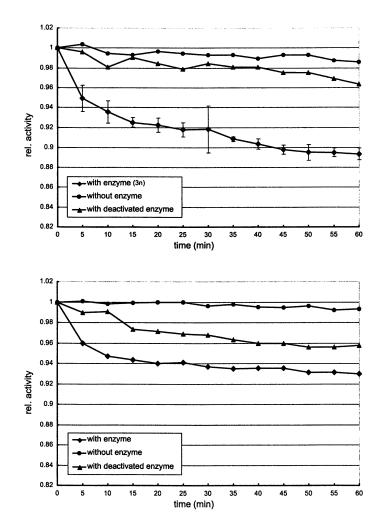


Figure 4. Time course of β -carotene (Top) and neoxanthin cleavage (Bottom) after treatment with crude enzyme preparation from rose flowers.

 β -Carotene was cleaved or oxidized by the treatment with crude enzymes during the incubation period of 60 min at an ambient temperature. Importantly, no evidence for autooxidation was observed and the heat-inactivated enzyme preparation did not oxidize β -carotene either. These results clearly indicate that the crude enzyme showed an oxidation activity toward β -carotene. The enzyme showed a weaker activity against neoxanthin (cf. Figure 4) under the same conditions. Since the heat-treated enzyme was also slightly active, further investigation is needed. Furthermore, it is necessary to analyze the incubation mixture to identify the reaction products. To the best of our knowledge, this is the first report on the detection of carotenoid-cleavage or oxidation enzyme in the flowers of *Rosa damascena*.

As suggested by Ohloff *et al.* (36) and Isoe *et al.* (37), an enzymatic cleavage of neoxanthin between C-9 and C-10 followed by the reduction of grasshopper ketone may result in a progenitor of 1. From the leaves of *Lycium halimifolium* (43), apple (*Malus domestica*) juice (44), and starfruit (*Averrhoa carambola* L.) (8), occurrence of damascenone 1 progenitors has been reported (Figure 5), i.e. allenic triol (2a) and its putative glycosidic compounds (2b, 2c, 2d). In rose flowers the genuine damascenone progenitor 2a has not been characterized up to now. This prompted us to isolate this key aroma progenitor from flowers of *R. damascena* Mill.

Isolation and Characterization of β-Damascenone Progenitors from Flowers of *R. damascena* Mill.

From the frozen flowers at full bloom stage, the progenitor fractions of 1 yielded β -damascenone 1 and 3-hydroxy- β -damascone 3 after heat treatment

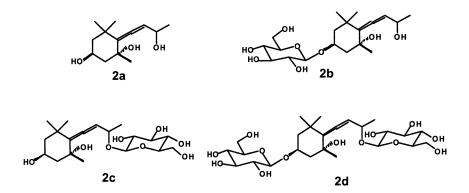
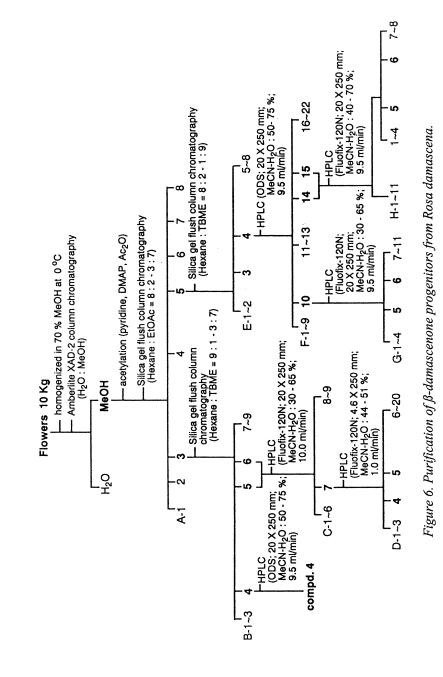


Figure 5. β-Damascenone precursor 2a and its putative glucosidic forms 2b-d.

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carbon	¹³ C (ppm)	¹ H ppm (mult., J in Hz)
1	35.9	
2	42.1	a, 1.55 (dd, 12.5, 3.0)
		b, 1.82 (ddd, 12.5, 3.5, 2.0)
3	67.8	5.00 (m)
4	37.3	a, 2.11 (dd, 17.5, 3.0)
		b, 2.47 (ddd, 17.5, 5.5, 2.0)
5	137.9	-
6	123.2	-
7	84.0	-
8	91.9	-
9	67.5	4.74 (q, 6.5)
10	23.1	1.51 (d, 6.5)
11	28.5	1.14 (s)
12	30.1	1.17 (s)
13	22.3	1.88 (br s)
1'	98.9	4.86 (d, 8.0)
2'	71.8	5.03 (dd, 9.5, 8.0)
3'	73.0	5.21 (dd, 9.5, 9.5)
4'	68.4	5.10 (dd, 10.0, 9.5)
5'	71.9	3.71 (ddd, 10.0, 5.0, 2.5)
6'	62.0	a, 4.09 (dd, 12.0, 2.5)
		b, 4.25 (dd, 12.0, 5.0)
5Ac	26.6~21.4	2.01 ~ 2.06 (5s)
	169.3 ~ 170.7	

Table 1. ¹H- and ¹³C- NMR data of 3-Hydroxy-7, 8-didehydro-B-ionyl 9-O-B-D-glucopyranoside pentaacetate.

under acidic conditions as suggested by Skouroumounis *et al.* (39). Guided by the detection of the ion peaks at $t_R = 29.0$ and 52.2 min for 1 and 3, respectively, each fraction was further purified as shown in Figure 6.

Beside the fraction A3-B4 from which precursor 4 was isolated at least three additional β -damascenone progenitor fractions (frs. A3-B5+6-C7-D-4+5, A5-E-4-F10-G-5+6, A5-E-4-F14+15) were obtained. Allenic triol 2a or its 3-O-glucopyranoside 2b, 9-O-glucopyranoside 2c, and 3,9-O-disaccharide glycoside 2d, respectively, were expected to be present in these fractions. Unfortunately these compounds were obviously not stable enough to be isolated. One of the progenitors was isolated and the chemical structure was elucidated to be 9-O-glucopyranosyl-3-hydroxy-7,8-didehydro- β -ionol (4) as a pentaacetate based on the CDI-MS, ¹H-¹H COSY, HSQC, and HMBC spectral data (cf. Table 1 and Figure 7). Compound 4 has been reported as a β -damascenone progenitor from the residual water after steam distillation of the flowers of *R. damascena* by Straubinger *et al.* (40). Sefton *et al.* (41) also reported the presence of the aglycone moiety of glucoconjugate 4 in wine.

In order to confirm the chemical structures of the progenitors, we tried to synthesize compounds **2a-d**. Compound **2b** was obtained as a diastereomeric mixture. To survey the presence of **2b** in the β -damascenone progenitor fractions, fr. A3-B5+6-C7-D-4+5 was analyzed by HPLC equipped with a photodiode array detector. However, glucoside **2b** could not be detected in this fraction. As already suggested by Skouroumounius *et al.* (*39*), substitution of aglycone **2a** with a β -D-glucosyl moiety retards the rate of dehydration at C-9 position. It may be important for the progenitors of 1 to be dehydrated at C-3 position prior to dehydration at C-9 position. The progenitor fraction, fr. A3-B5+6-C7-D4+5 is therefore expected to contain 9-O- β -D-glucopyranoside (**2c**) instead of **2b** as a progenitor of 1. Chemical synthesis of **2c** and **2d** is still under active investigation.

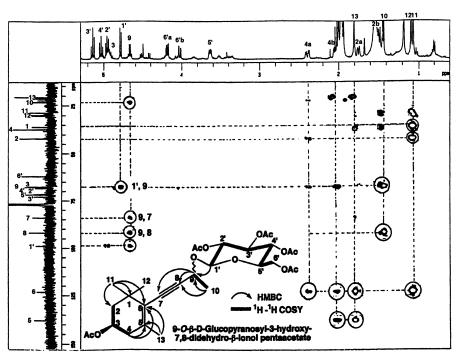


Figure 7. HMBC spectrum and structure of pentaacetate of compound 4 (CDCl₃, 500 MHz)

Summary

The presence of non-volatile progenitors of β -damascenone (1) in rose flowers, *Rosa damascena* Mill. was studied. We found several carotenoids such as β -carotene, zeaxanthin, and neoxanthin in the flowers, with the latter being able to be enzymatically oxidized and cleaved to possible progenitors of 1. We have also succeeded in isolating and characterizing 9-O-glucopyranosyl-3hydroxy-7,8-didehydro- β -ionol 4 as an important progenitor of 1.

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Chapter 8

Production of Aroma Compounds by Enzymatic Cooxidation of Carotenoids

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A process for enzymatic co-oxidation of carotenoids was developed involving the action of xanthine oxidase on an aldehyde or purine to produce free radicals. The kinetics of the oxidation was depending on the free radicals generated, themselves depending on the enzymatic substrate, and on the stucture of the starting carotenoid. This process was very efficient for the production of β -ionone, epoxy- β -ionone and dihydroactinidiolide from β -carotene and it was also possible to cleave the C9-C10 bond of neoxanthin to give precursors of damascenone including grasshopper ketone.

Carotenoid-derived aroma compounds are of great interest to the food and perfume industry. However, their extraction from natural products is often difficult and expensive. In this area as in other fields of natural products production, research has developed processes copying nature in order to avoid or to complete chemical synthesis while increasing production yields. Among these ways of carotenoid oxidation, enzymatic processes can have a real interest due to substrate specificity and reduced production time. In this study, cooxidation of carotenoids by enzymatic free radical generation will be discussed.

Co-oxidation System

Many processes are dealing with carotenoids oxidation including the use of oxidants or thermal treatments. Enzymatic oxidation systems have also been developed as for example co-oxidation, i.e. carotenoids are oxidized by free radicals generated from another substrate through an enzyme action. Co-oxidation of β -carotene using various enzymes (1,2), especially lipoxygenase (3,4) has been extensively studied. The objective of these co-oxidation experiments was the indirect measurement of enzyme activity or the identification of oxidation products from β -carotene. In some cases, the purpose was to produce aroma compounds such as β -ionone. Some studies on the production of volatiles were carried out in our laboratory (2,5) leading to a development process (6). We then decided to study more precisely the mechanisms taking part in this system using the enzyme xanthine oxidase (7-9).

Xanthine Oxidase

Xanthine oxidase is a flavoprotein catalysing oxidation of xanthine to uric acid and superoxide anion. The enzyme is not substrate specific and it can oxidize more than a hundred compounds including many aldehydes. The mechanism of aldehyde oxidation is the following (7):

(1)
$$R-CHO + H_2O \rightarrow R--CH(OH)_2$$

(2)
$$R$$
—CH(OH)₂ + Enzyme \rightarrow R—COOH + Enzyme—H₂

(3) Enzyme— $H_2 + O_2 \rightarrow Enzyme + 2 H^+ + O_2^{\bullet}$

Aldehydes are more soluble and cheaper than xanthine or other purine. For that reason, we tested acetaldehyde, propanal and butanal. Resulting values for Km were equal to $3.2.10^{-3}$, $1.6.10^{-2}$ and $3.5.10^{-2}$ M, respectively. The optimal pH value are 7.8 for xanthine and 8 for acetaldehyde and the activity is twice as high at 37 °C than at 25 °C (10). Oxygen has to be present in the medium as it is the co-substrate. The process conditions have to take into account that xanthine oxidase can be inhibited by excess of substrate (aldehydes or xanthine) (11) or by the reaction products like O₂[•] or the carboxylic acid, especially in diluted media.

Free Radical Production

As shown in equation (3), aldehyde oxidation generates O_2^{\bullet} . This highly reactive species will give rise to other radical species depending on the nature of the co-substrate. Detection and identification of free radicals were carried out using electron spin resonance (7,8). Superoxide anion was always detectable and - due to its reactivity – formation of additional radicals was observed (Table 1). From aldehydes, alkyl radicals were formed including oxygenated radicals for acetaldehyde.

Substrate	Free radicals
Acetaldehyde	O ₂ [•] , (HO [•]), [•] CH ₃ , [•] CH ₂ OH, CH ₃ O [•] and CH ₃ OO [•]
Propanal	O_2^{\bullet} , (HO [•]), C_2H_5
Butanal	O_2^{\bullet} , (HO [•]), C_3H_7
Xanthine	$O_2^{\bullet,}$ (HO [•])

Table 1.	Free Radicals	Detected for	Various	Substrates

SOURCE: Data are from reference 7

Medium Preparation

Defining an efficient reaction system for co-oxidation implies good enzymatic conditions for free radical production as well as good access to the carotenoid. The first point could be achieved in an aqueous system at a pH value around 8 and at 37 °C which corresponds to the best enzymatic activity and to an acceptable O_2 solubility. In order to increase oxygen availability, the reaction was carried out under air pressure.

The second point will depend on the solubility of the carotenoid. β -Carotene is insoluble in water but very soluble in non-polar solvents. Application of a biphasic system with carotene dissolved in the oil phase is possible but will also generate numerous fatty-acid derived oxidation products. The best way to solubilize carotene was achieved by the use of Tween 80 (12). Carotene and Tween 80 were dissolved in chloroform which was evaporated, EDTA was added and the reaction medium was ready for the co-oxidation experiment. Under those conditions, the average diameter of the hydrophobic particles as measured with a granulometer was 0.5 µm. Details of experimental procedures have been described elsewhere (7-9). Application of a biphasic system with enzyme containing reversed micelles has been tested, but contrary to lipoxygenase, xanthine oxidase lost its activity under these conditions (13).

Co-oxidation of β-Carotene

The co-oxidation system was studied first with synthetic β -carotene (98 % all-*trans*). In this case, the main products were β -ionone, epoxy- β -ionone and dihydroactinidiolide. This system was used as a model to study the impact of free radicals and also of *cis-trans* isomerism of the carotenoid on the product yields.

Kinetics of β-Carotene Degradation and Main Products Appearance

The bleaching of β -carotene (Figure 1) was the first visible proof of oxidation. Within 2-4 h, the orange solutions turned either to pale yellow or almost colorless solutions when low initial concentrations were used. In a blank experiment the tendency of β -carotene to auto-oxidize was recorded. For the initial β -carotene solution of 10 mg/l, almost 100 % was degraded after only two hours. The action of xanthine oxidase was particularly evident during the first 1.5 hour. Afterwards, the degradation kinetics for β -carotene was comparable to the blank.



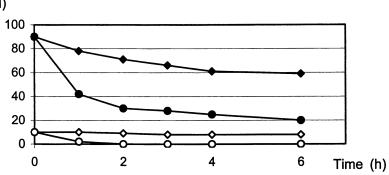


Figure 1. Degradation of β -carotene in auto-oxidation (\blacklozenge , \Diamond) and co-oxidation (\blacklozenge , \bigcirc) systems. Co-oxidation experiments were carried out with xanthine oxidase and 90 mg/l (full symbols) or 10 mg/l (open symbols) of β -carotene as the initial concentration and acetaldehyde as the substrate. (Adapted with permission from reference 8. Copyright 1994 American Chemical Society)

GC/MS allowed us to identify many volatiles formed in the solutions. After the first hour of incubation, it was already possible to detect the substances

presented in Table 2. Other compounds have been identified including lactones and epoxides of degraded β -carotene.

Туре	Substances
Aldehyde	geranial
	neral
	β-cyclocitral
Ketone	β-ionone
	5,6-epoxy-β-ionone
	dihydroactinidiolide
	pseudoionone

 Table 2: Main Substances Identified After 1 h of Enzymatic Degradation of

 β-Carotene

The kinetics of β - and 5,6-epoxy- β -ionone production is shown in Figure 2. We noticed an one-hour shift between the concentration maxima for the two ionones. The two molecules were degraded very quickly after two or three hours and the difference in time of maximal concentration showed that epoxy- β ionone could be an oxidation product from β -ionone. Dihydroactinidiolide appeared also after only one hour of reaction, but the formation continued for three hours. It could be formed from epoxy- β -ionone as presented in Figure 3.

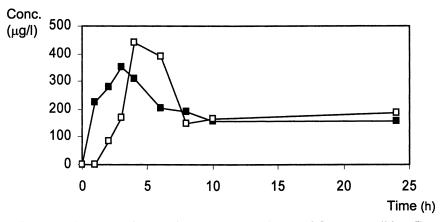


Figure 2. Aroma production during the co-oxidation of β -carotene (90mg/l) with 48 mM acetaldehyde and 27 × 10⁻³ unit/ml xanthine oxidase: β -ionone (\blacksquare) and epoxy- β -ionone (\Box) (Adapted with permission from reference 8. Copyright 1994 American Chemical Society)

Effect of Free Radicals

As shown above, the various primary xanthine oxidase substrates generated different free radicals. Kinetics of β -carotene degradation was therefore studied with different substrates. We noticed that for xanthine, the kinetics of carotene degradation was comparable with auto-oxidation: the superoxide anion was probably inactive on carotene. On the other hand, all of the tested aldehydes, i.e. acetaldehyde, propanal, butanal, para-hydroxybenzaldehyde and benzaldehyde were active. The most active species were those generated by acetaldehyde and butanal. Although butanal degraded the co-substrate more rapidly, the production of volatiles was slower.

Although O_2^{\bullet} is obviously not active towards β -carotene, it is able to generate other free radicals depending on the substrate. Most likely, the generated radicals then degrade the carotene molecule. The action could depend on the actual size of the radicals and on steric hindrance: free radicals from butanal are longer and less reactive molecules than radicals from acetaldehyde. However, after four hours, the butanal experiment leads to good product yields probably due to a slower degradation of reaction products.

Effect of E/Z Isomerism of β-Carotene

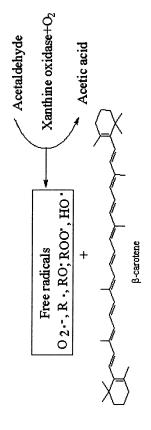
For β -ionone production, the reactivity of the C9-C10 bond of β -carotene is important and this reactivity depends on *cis-trans* isomerism. Although 272 geometric isomers of β -carotene theoretically exist, only 12 *cis*-compounds in total have been recorded and analyzed (14). The most common are *mono* 7-, 9-, 13- and 15-*cis*- β -carotene and some poly-*cis* isomers like 9,9'-di-*cis*- β -carotene.

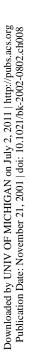
In order to compare the different isomers, we used Advanced Molecular Design (Oxford Molecular) to calculate the most probable conformation for each molecule in vacuum (15). It was then possible to calculate the theoretical ΔG for carotene oxidation reactions leading to small aldehydes. The model results confirm the effect of *cis-trans* isomerism on the oxidation reaction products as the favored products are different for the various isomers. In theory, favored scission sites are C7-8 for all-*trans*- β -carotene and C9-10 for 9-*cis* β -carotene.

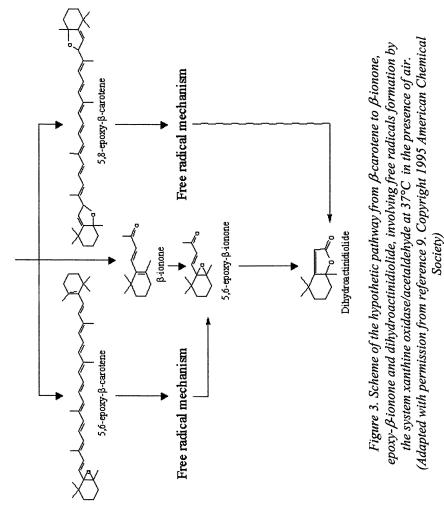
Favored products for all-*trans*- β -carotene (from highest to the lowest probability of appearance): β -cyclocitral > β -ionone > apo-13-carotenone > β -ionylidene acetaldehyde. For 9-*cis* β -carotene: β -ionone > β -cyclocitral > 9-*cis*- β -ionylidene acetaldehyde.

To confirm these predictions, we tested experimentally the degradation of preparations with increased 9-*cis* compounds concentration (Table 3). Apart from the synthetic all-*trans*- β -carotene, we used a preparation generated by an

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iodine-catalyzed photoisomerization of all-*trans*- β -carotene (16) and a commercial preparation of carotenoids from the green algae *Dunaliella salina* naturally rich in the 9-*cis* compound (Betatene®, Henkel, La Grange, IL).

Form	Synthetic &carotene	Iodine catalyzed photo- isomerized	betatene
All-trans	96 %	54 %	58 %
9- <i>cis</i>	0.8 %	25 %	42 %
13- <i>cis</i>	1.3 %	19 %	-
15- <i>cis</i>	1.9 %	2 %	-

Table 3: Proportion of Isomers in Various β-Carotene Mixtures as Determined by HPLC.

SOURCE: Data are from reference 7.

In the mixtures tested, the proportion of the 9-cis form varied from 0.8 to 42.0 %. By using the different preparations, the influence of isomerism on the degradation kinetic and on the oxidation products was studied. The proportion of the different isomers did not vary significantly, neither during the co-oxidation experiment nor during the auto-oxidation (results not shown). This shows that *cis*-isomers are not more fragile than all-*trans* under the tested conditions. We could not see any difference in the concentrations of the reaction products either. This points out the differences between the model and the experiment as the model has been calculated in vacuum taking into account oxidation by O_2 whereas the experiment involves the solubilization of carotene by Tween and the attack by free radicals. As there were no differences between the dispersion procedure to protect the whole molecule of carotene including the *cis*-bond.

Co-oxidation of Neoxanthin

Damascenone 5 (cf. Figure 4) is an important flavor compound, first identified in Bulgarian rose (*Rosa damascena*) oil in 1970 (17). Its smell is green and sweet, like raspberry, rose or whisky. Other parts of this book discuss this molecule confirming its great importance in the world of aromas. Despite the importance of ketone 5, no biotechnological production method exists. β -Damascenone 5 production requires an allenic precursor with the right carbon skeleton such as neoxanthin 1. Cleavage of this molecule gives rise to damascenone precursors like grasshopper ketone 2 or the allenic triol 3 (see Figure 4).

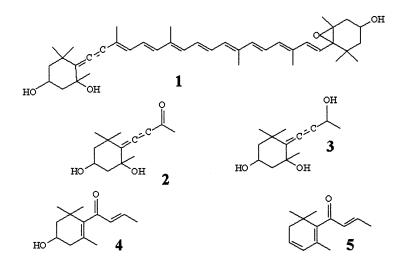


Figure 4. Structure of neoxanthin 1, β -damascenone 5 and the intermediates of the pathway proposed by Isoe et al.(18): grasshopper ketone 2, allenic triol 3 and 3-hydroxydamascone 4.

The degradation system was very efficient also for substrate 1. With xanthine oxidase, 90 % of neoxanthin were degraded in the first hour whereas the degradation was slow for auto-oxidation. Many degradation products were encountered. These products included norisoprenoids like grasshopper ketone, 3-hydroxy- β -ionol, 3-oxo- β -ionol, β -ionone and β -ionol.

Despite the significant effect of xanthine oxidase on the speed of neoxanthin degradation (Figure 5), co-oxidation and auto-oxidation products were qualitatively not different. Only differences with regard to the concentration of the reaction products were observed. In both cases, the C9-C10 cleavage site was favored giving rise to potential precursors of damascenone. But the C7-C8 scission site was also important since a lot of smaller, unidentified molecules were also detected. Neoxanthin degradation in %

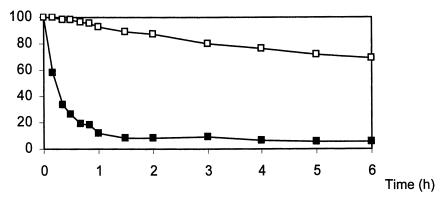


Figure 5. Neoxanthin degradation in percent vs time: co-oxidation with xanthine oxidase and acetaldehyde (\blacksquare) and auto-oxidation (\Box). (Data from reference 19)

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Chapter 9

Thermal Generation of Carotenoid-Derived Compounds

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The kinetics of formation of volatile and nonvolatile compounds during heat treatment of B-carotene and lycopene suspension in water were determined for several temperatures and oxygen content. The precursor of the first compound produced during the thermal treatment of B-carotene in the presence of oxygen, i.e. dihydroactinidiolide, was identified by chromatography, UV-, ¹H-NMR- and IR-spectroscopy to be mutatochrome. Dihydroactinidiolide was also generated from 5,6-epoxy-B-ionone which can be considered as an important intermediate involved in the formation of 2-hydroxy-2,6,6trimethylcyclohexanone and 2-hydroxy-2,6,6-trimethylcyclohexane-1-carboxaldehyde. 2-Methyl-2-hepten-6-one, geranial and pseudoionone can be considered as the primary degradation products resulting from the oxidative cleavage of the 6-7, 8-9, and 10-11 bonds of lycopene. 6-Methyl-3,5heptadien-2-one and dihydropseudoionone or geranyl acetone can be formed from pseudoionone, whereas the cis-trans isomerization of geranial gives neral.

Carotenoids are yellow, orange or red tetraterpene pigments responsible for the natural color of several feedstuffs and foods. They are synthesized by fruits

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and vegetables, algae and bacteria and are accumulated from these sources in some animal tissues, like sea foods, eggs or some birds like flamingos. In the latter case, the pigments produced by the alga *Dunaliella* are accumulated by a micro shrimp (*Mona dunalis*) which is eaten by the flamingos. Several carotenoids such as β -carotene, lycopene, canthaxanthin or bixin obtained by extraction from natural sources or by synthesis are used as food colorants.

In plants, carotenoids are associated with chlorophyll in the chloroplasts. The result of their synthesis during ripening of fruits and degradation of chlorophyll is the change of chloroplasts to chromoplasts. Different mechanisms are involved in the degradation of these unsaturated, labile structures, such as, e.g., isomerization, bleaching or cleavage of several bonds located at the central region of the molecule to give nonvolatile and volatile compounds.

In vivo, at the onset of senescence, several aroma compounds are produced by photooxygenation of β -carotene, as suggested by the formation of β -ionone and dihydroactinidiolide from this compound (1). More recently, evidence for the *in vivo*-formation of norisoprenoid compounds by enzymatic action of yet unidentified dioxygenase systems was given. For example, Güldner and Winterhalter (2) showed that the absolute configuration of 3-hydroxy- β -ionone found as a glycosidically bound component in quince fruit was the same as found in the parent carotenoid zeaxanthin.

The enzymatic bleaching of plant pigments, more particularly the cooxidation of β -carotene, canthaxanthin or crocin, was the result of the action of polyphenol oxidase (3), as well as lipoxygenase in the presence of unsaturated fatty acids (4). Lactoperoxidase (5) and xanthine oxidase (6) are also involved in this reaction. However, studies devoted to the isolation and identification of volatile compounds generated during the process are scarce. Only five compounds, i.e. 3,6,6-trimethylcyclohexanone, 2-hydroxy-3,6,6-trimethylcyclohexanone, β -ionone, 5,6-epoxy- β -ionone and dihydroactinidiolide were identified (7-9). Peroxyl radical autoxidation, chemical oxidation and singlet oxygen attack of carotenoid pigments were also studied (10,11). Several aroma compounds previously identified among β -carotene degradation products were generated by chemical oxidation of this pigment, such as, e.g., the canthaxanthin metabolite 4-oxo- β -ionone.

Another important mechanism involved is the thermal degradation of carotenoids which is the subject of the present chapter.

Thermal Degradation of Food Carotenoids

The development of a 'violet-like' off-flavor attributed to β -ionone formed from β -carotene during dehydrated carrot storage was reported by Tomkins et al. (12). Several volatile compounds such as β -ionone, 5,6-epoxy- β -ionone, dihydroactinidiolide, β -cyclocitral, which were identified after heat treatment of plant products, such as tea, tobacco, passion fruit, grapes, apricot, mango or algae (13-22), were considered as thermal degradation products of carotenoids. A 37 % loss of carotenoids (relative to the blank) during the heat treatment of mango pulp at 95°C (10 min) resulted in an increase of the concentration of dihydroactinidiolide (DHA) and β -ionone (cf. Figure 1). These results indicated that dihydroactinidiolide and β -ionone were produced by thermal degradation of β -carotene which is the main carotenoid in mango (20). The increase of damascenone content after heating was probably the result of an acid-catalyzed conversion, at natural pH of the fruit, of the allenic triol resulting from the enzymatic reduction of grasshopper ketone, the latter being a primary oxidative cleavage product of neoxanthin (23). Neoxanthin was recently found in small amounts in the mango cultivar Keitt (24). Rearrangement of either luteoxanthin or violaxanthin or of degradation compounds, such as 3-hydroxy- β -ionone, is also considered as being likely.

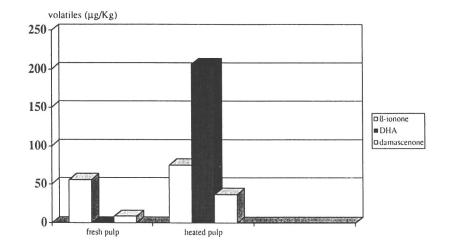


Figure 1. Volatile compounds ($\mu g/Kg$ of pulp) formed by thermal degradation (95°C, 10 min) of β -carotene from an African mango.

Moreover, a 60% decrease of xanthophyll content was observed when the mango pulp was heated at 100°C for 1 to 3 h. HPLC analyses indicated a significant decrease of luteoxanthin, the main xanthophyll present in this mango variety. This decrease in luteoxanthin concentration was the result of both, its degradation as well as the rearrangement of the 5,6-epoxy group into a 5,8-epoxy group, as indicated by NMR measurements.

2-Methyl-2-hepten-6-one and - at a lesser degree - geranyl acetone and citral were described by several authors as degradation products of lycopene in tomato juices and tomato paste (25-27). According to these authors, thermal or enzymatic pathways are involved in the formation of these volatiles. The formation of 2-methyl-2-hepten-6-one and of geranyl acetone during tomato paste processing is connected with a decrease in the lycopene content (Table I).

Table I. Formation of Volatile Compounds by Degradation of Lycopene
During Tomato Paste Processing

Product	Lycopene (mg/kg)	2-Methyl-2-hepten-6-one (µg/kg)	Geranyl acetone (µg/kg)
Fresh tomato	118	147	43
Tomato paste	73	466	135

Several thermal degradation products from ascidian tunic carotenoids, such as carotene, zeaxanthin, astaxanthin, fucoxanthin and canthaxanthin were identified after heating at 100°C for 3 hours (28). They included B-ionone, dihydroactinidiolide, 3,5,5-trimethyl-3-cylohexen-1-one, 1,1,2,3-tetramethyl-2cyclohexen-5-one and the corresponding alcohols, as well as 2,3,4,4-tetramethyl-6-hydroxy-2-cyclohexen-1-one and 1,2,3,8-tetrahydro-3,3,6-trimethyl-1-naphthol.

Carotenoid Thermal Degradation in Nonaqueous Medium

The different hypotheses formulated concerning the thermal formation of volatile cleavage products from food carotenoids are founded on experimental results obtained from carotenoid degradation studies employing model systems. According to the high hydrophobicity of most of the carotenoids, several studies were performed in nonaqueous media. Organic solvents, such as benzene, toluene, dichloromethane, n-dodecane, hexane, ethanol or glycerol were used at temperatures varying from 60-220°C. Reaction times were 10 min to 72 h or longer (1-5 days) and the reaction was carried out in the presence of oxygen, air or nitrogen (10,29-36). In some cases, the pigments were heated in solid state (8,37).

The presence in the volatile fraction of aromatic compounds (e.g. toluene, m-xylene) is reported by several authors (29,30). More interesting is the identification – of varying amounts - of ionene, 2,6-dimethylnaphthalene, β -

cyclocitral, β -ionone, 5,6-epoxy- β -ionone, dihydroactinidiolide and 3-oxo- β ionone. β -Carotene in deoxygenated n-dodecane or hexane was thermolyzed at 170-200°C (15-18 h up to 1-5 days) in order to understand the origin of the aromatic fraction of petroleum (32). Several polyenes and volatile compounds were formed under the experimental conditions applied. Aromatic volatiles included toluene, o-, m-, and p-xylene, 1,1,3-trimethylcyclohexane and 1methyl-3-ethylbenzene. Two colorless polyenes, 3,7,10-trimethyl-1,1,12-bis (2,6,6-trimethylcyclohex-1-enyl)dodeca-1,3,5,7,9,11-hexaene and 3,6-dimethyl-1,8-bis(2,6,6-trimethylcyclohex-1-enyl)octa-1,3,5,7-tetraene were tentatively identified when β -carotene was heated in glycerol at 210°C for 4 h (31). The authors stated that the formation of these compounds was the result of the thermal loss of toluene from the central part of the polyene chain, a mechanism for the formation of the former compound was also proposed.

To the best of our knowledge, only one work (8) was devoted to the formation of aroma compounds from lycopene degradation. In this study, 2-methyl-2-hepten-6-one was identified after thermal treatment of lycopene at 190 and 220°C. The heating of canthaxanthin at 210°C for 30 to 40 minutes under nitrogen resulted in a loss of 87-100 % of this pigment. Six oxygenated volatile compounds were tentatively identified by GC-MS, i.e. 4-methyl-3-penten-2-one, 2,4,4-trimethyl-2-cyclohexen-1-one, 1,1,5,6-tetramethyl-4-oxo-5-cyclohexene, 1,2,3,4-tetrahydro-4-oxo-1,1,6-trimethylnaphthalene, 2-(1,1,5-trimethyl-4-oxo-5-cyclohexen-6-yl)-1-tolylethene, and 2,6-dimethyl-8-(1,1,5-trimethyl-4-oxo-5-cyclohexen-6-yl)-1,3,5,7-octatetraene.Two mechanisms for the formation of 1,2,3,4-tetrahydro-4-oxo-1,1,6-trimethylnaphthalene from canthaxanthin were postulated (*36*).

However, the above mentioned reactions generally occurred under more drastic temperatures and longer reaction times than those usually applied for thermal treatment of foods.

Carotenoid Thermal Degradations in Aqueous Medium

 β -Carotene or lycopene suspended by sonication in distilled water in a sealed Kjeldahl flask wrapped in aluminum foil was heated for 1-4 hours at 30 to 97°C in the presence of air, oxygen or nitrogen. The volatile compounds formed by thermal degradation of β -carotene at 97°C for 3 h under different atmospheres (cf. Figure 2) indicated the oxidative nature of the process. It was found that - in oxygen atmosphere - kinetics of degradation of the pigment was a zero order reaction, as previously reported by El Tinay and Chichester (10) for the degradation in toluene solution.

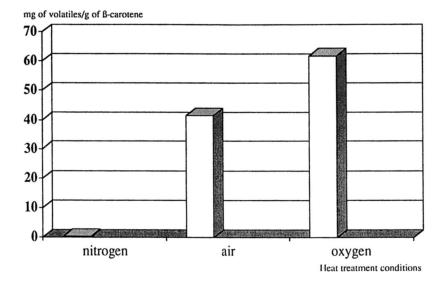


Figure 2. Total amount (mg/g of β -carotene) of volatile compounds formed during thermal degradation of β -carotene (3 h, water suspension, 97°C) under nitrogen, air, and oxygen, respectively.

Nonvolatile Compounds

The nonvolatile compounds produced during the heat-treatment of β carotene were separated by preparative TLC and their spectral characteristics were determined. UV spectra and staining by hydrochloric acid treatment indicated the presence of aurochrome, mutatochrome, 5,6-epoxy- β -carotene, and neo-T-5,6,5',6'-diepoxy- β -carotene (38). Moreover, an oxo-polyene with λ_{max} at 389 and 401 nm was also detected. Confirmation of these identifications was given by NMR spectroscopy at 360 MHz. According to previously published data (39), singlets at 1.10, 1.14, 1.48 and 1.84 ppm are indicative of the presence of methyl groups close to an 5,8-epoxide ring (compound tentatively identified as aurochrome). Similarly, methyl singlets at 0.93 or 0.98, 1.14 and 1.14 or 1.15 ppm are characteristic of 5,6-epoxides (compounds tentatively identified as 5,6-epoxy- β -carotene and 5,6,5'6'-diepoxy- β -carotene) (38).

Vibration	Aurochrome (cm ⁻¹)	neo-T-5,6,5',6'- diepoxy- β-carotene (cm ⁻¹)	5,6-β-epoxy- β-carotene (cm ⁻¹)
ν C-H	3020	2920, 2970, 2850	2850, 2920
ν=C-H	2920, 2850	3025, 3060	3020
v –CO	1030, 1150	1070, 1150	1070, 1150
Cis δ CH ₃ -CH=CH-	1375	1360	1375
Cis δ -CH=CH-	760, 790	760, 790, 1451	760, 790, 1451
Trans δ -CH=CH-		980	1025

Table II. Infrared Identification of Nonvolatile Compounds Produced After Thermal Degradation of B-Carotene (97°C for 3 h)

The results of infrared spectroscopy (Table II) were in accord with data obtained by the other spectroscopic methods. More particularly the low intensity of the trans CH=CH vibration band at 980 cm⁻¹ relatively to those of the cis - CH=CH vibrations (760, 790, 1451 cm⁻¹) indicated that 5,6,5',6'-diepoxy-ß-carotene is the neo-T isomer (40).

Five epoxides namely 5,6-epoxy- β -carotene, 5,8-diepoxy- β -carotene or mutatochrome, 5,6,5',6'-diepoxy- β -carotene, 5,6,5',8'-diepoxy- β -carotene or luteochrome, and 5,8,5',8'-diepoxy- β -carotene or aurochrome were generated during heat treatment of all trans- β -carotene at 180°C for 2 h in dichloromethane (33). These epoxides were also found after extrusion cooking of a starch- β -carotene mixture at 180°C. Moreover five apocarotenals and β -carotene-4-one were formed by oxygen attack on the polyene chain depending on the nature of the model system used (for details cf. ref. 34). Most of the above mentioned compounds were detected after spontaneous autoxidation of toluene solutions of β -carotene with 100 % of oxygen at 60°C for one hour (41).

The formation of nonvolatile compounds at 97°C in the presence of air or oxygen was followed over increasing reaction time by estimation of the intensity of the different spots obtained by TLC. In these two cases, considerable amounts of 5,6-epoxy- β -carotene, and 5,6,5',6'-diepoxy- β -

carotene, and small amounts of mutatochrome were detected after one hour of reaction (38). Spots corresponding to aurochrome and oxopolyene were observed after a reaction time of 2 hours. The intensity of the spots corresponding to these compounds increased except the spot for 5,6-epoxy- β -carotene which remained constant. The kinetics of formation of nonvolatile compounds in the presence of oxygen is faster than the one of the same compounds in the presence of air. These results confirmed the importance of oxygen concentration on the degradation of β -carotene.

It can be postulated that the first compounds produced during the thermal degradation of β -carotene were 5,6-epoxy- β -carotene and mutatochrome. The first compound is rapidly oxidized to 5,6,5',6'-diepoxy- β -carotene whereas mutatochrome gave ultimately aurochrom. Luteochrome identified by Marty and Berset (33) is likely to act as a transient intermediate between these two compounds. In the case of lycopene, only all trans and cis-trans lycopene were identified after the thermal degradation of the pigment at 97°C (1-3 h) in the presence of air or oxygen (42).

Volatile Compounds

In addition to previously reported volatile compounds of thermal β -carotene degradation decanal, 4-oxo- β -ionone, β -methyl ionone and ketoisophorone were found. Trimethyldecahydronaphthalene (1), 2-hydroxy-2,6,6-trimethylcyclohexanone (2) (38), actinidol (3), and two isomers of 1-(1,2,2-trimethyl-3-cyclopentenyl)-1-pent-2-en-1,4-dione (4,5) were tentatively identified as degradation products of β -carotene (cf. Figure 3). The most abundant were 5,6-epoxy- β -ionone and dihydroactinidiolide. Additional compounds included 2-hydroxy-2,6,6-trimethylcyclohexane-1-carboxaldehyde, 4-oxo- β -ionone, β -cyclocitral, 2-hydroxy-2,6,6-trimethylcyclohexanone, and β -ionone. Other compounds were only present in minute quantities. Our results were in agreement with those previously reported by Kawakami (43).

The kinetics of formation of dihydroactinidiolide from β -carotene heated at 97°C during 0.5-6 h in the presence of air or oxygen (cf. Figure 4), indicated that the concentration of dihydroactinidiolide increased with reaction time and oxygen content of the medium. Hence, it can be assumed that this compound was one of the first volatile compounds formed by direct cleavage of the 8,9bond of aurochrome and/or mutatochrome. Morever dihydroactinidiolide was not the subject of ultimate cleavages or rearrangement reactions. Conversely, the concentration of 5,6-epoxy- β -ionone (cf. Figure 5) increased within the first two hours of heating and then decreased, whereas the levels of β -ionone and 2-hydroxy-2,6,6-trimethylcyclohexane-1-carboxaldehyde increased between 0.5 to 4 hours.

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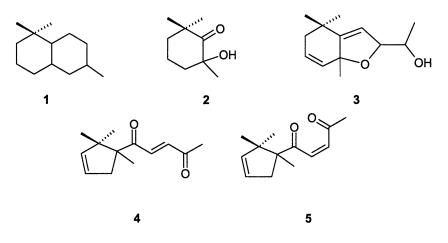


Figure 3. Volatile compounds tentatively identified during thermal degradation of β -carotene (suspension in water, 97°C, 3 h): Trimethyl-decahydronaphthalene (1), 2-hydroxy-2,6,6-trimethylcyclohexanone (2), actinidol (3), trans- and cis- isomers of 1-(1,2,2-trimethyl-3-cyclopentenyl)-1-pent-2-en-1,4-dione (4,5).

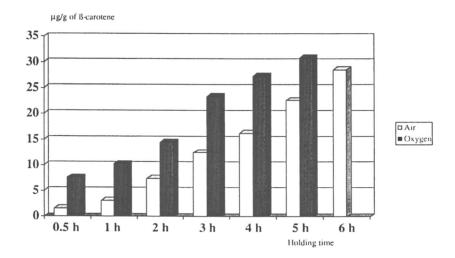


Figure 4. Kinetics of formation of dihydroactinidiolide during thermal degradation of β-carotene (suspension in water, 97°C, different holding times) in the presence of air and oxygen, respectively.

In Carotenoid-Derived Aroma Compounds; Winterhalter, P., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2001. 123

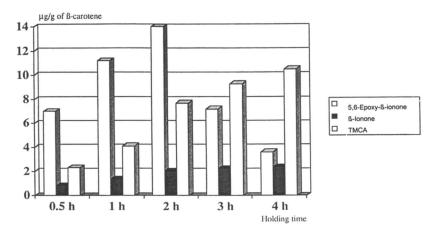


Figure 5. Kinetics of formation of 5,6-epoxy-ionone, β -ionone and 2-hydroxy-2,6,6-trimethylcyclohexane-1-carboxaldehyde (TMCA) during thermal degradation of β -carotene (suspension in water, 97°C, different holding times) in the presence of oxygen.

It appears that similar to dihydroactinidiolide 5,6-epoxy- β -ionone is also produced at the beginning of the degradation process by cleavage of the 9,10 double bond of 5,6-epoxy- β -carotene and/or of the 9,10 (9',10') double bonds of 5,6,5',6'-diepoxy- β -carotene (Figure 6). The kinetics of 5,6-epoxy- β -ionone formation suggests that this compound was the precursor of 2-hydroxy-2,6,6trimethylcyclohexane-1-carboxaldehyde. 2-Hydroxy-2,6,6-trimethylcyclohexannone and dihydroactinidiolide were probably also produced from epoxy- β ionone. The conjugated β -cyclocitral and 2,6,6-trimethyl-2-cyclohexen-1-one were the result of the dehydration of the hydroxy aldehyde and of the hydroxy ketone, respectively. Likely pathways giving rise to 2,6,6-trimethylcyclohexanone, 4-oxo- β -ionone and ketoisophorone were proposed on structural basis only.

Compounds obtained after action of superoxide anion, generated by action of xanthine oxidase on acetaldehyde (pH 8, 39°C) during 24 h were identified as 5,6-epoxy- β -ionone and small amounts of aurochrome. The major volatile compounds identified were essentially the same as those resulting from thermal

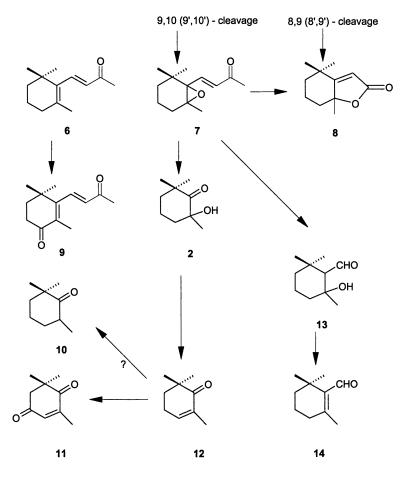


Figure 6. Postulated mechanism of formation of major volatile compounds upon thermal degradation of β-carotene (suspension in water, 97°C, 3 h): β-ionone (6), 5,6-epoxy-β-ionone (7), dihydroactinidiolide (8), 4-oxo-β-ionone (9), 2-hydroxy-2,6,6-trimethylcyclohexanone (2), 2,6,6-trimethylcyclohexanone (10), ketoisophorone (11), 2,6,6-trimethyl-2- cyclohexen-1-one (12), 2-hydroxy-2,6,6-trimethylcyclohexane-1-carboxaldehyde (13), and β-cyclocitral (14).

degradation of β -carotene. These results indicated that the mechanisms involved were similar in both cases. Formation of β -ionone and β -cyclocitral was not observed after singlet oxygen degradation of β -carotene in the presence of rose bengal during 24 h at 20°C. This indicates a different mechanism of singlet oxygen attack on β -carotene. Conversely, β -ionone was found to be the first volatile compound generated by co-oxidation of β -carotene using lipoxygenase from soybean in the presence of linoleic acid (9).

Volatile compounds isolated after heat treatment of lycopene at 97° C for 1 and 3 h in the presence of oxygen are listed Table III (42). The most abundant compounds were 2-methyl-2-hepten-6-one, pseudoionone, geranial and 6-methyl-3,5-heptadien-2-one. Three of these compounds were detected after heat treatment of lycopene at only 50°C (42). They can be considered as primary compounds resulting from the oxidative cleavage of the 6-7, 8-9 and 10-11 bonds, respectively (cf. Figure 7). 6-Methyl-3,5-heptadien-2-one and dihydropseudoionone (or geranyl acetone) can be formed from pseudoionone, whereas the trans-cis isomerization of geranial gave neral. The mechanisms involved in the formation of 5-hexen-2-one and hexane-2,5-dione from 2-methyl-2-hepten-6-one were not clearly established.

Analogous results to those found for β -carotene were obtained for superoxide anion degradation of lycopene, whereas the identification of farnesene among the volatile compounds generated after photochemical treatment indicated again a clearly different degradation mechanism for singlet oxygen attack.

Volatile compounds	Conc. (µg/100g) 1 h	Conc. (µg/100g) 3 h
5-hexen-2-one	1 1	0.31
2-methyl-2-hepten-6-one	4.2	8.6
hexane-2,5-dione	-	0.34
6-methyl-3,5-heptadien-2-one	0.47	4.5
neral	0.27	1.3
geranial	1.5	4.3
geranyl acetone	-	0.26
pseudoionone ^t	0.65	3.0

Table III. Volatile Compounds Generated During Heat Treatment of	ľ
Lycopene (97°C, 1-3 h) in the presence of oxygen	

^ttentatively identified

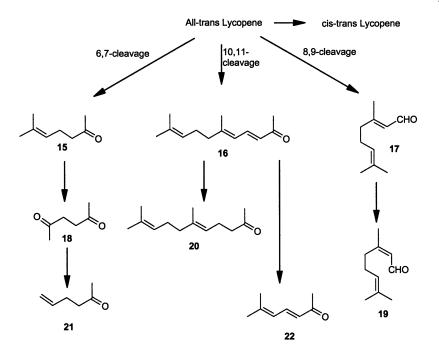


Figure 7. Postulated mechanism of formation of the main volatile compounds during thermal degradation of lycopene (suspension in water, 97°C, 3 h): 2methyl-2-hepten-6-one (15), pseudoionone (16), geranial (17), hexane-2,5dione (18), neral (19), geranyl acetone (20), 5-hexen-2-one (21), and 6-methyl-3,5-heptadien-2-one (22).

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Chapter 10

Carotenoid-Derived Aroma Compounds in Tobacco

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More than one hundred compounds, classified as degraded carotenoids, have been isolated from tobacco. These have 15, 13, 12, 11, 10, or 9 carbon atoms and are oxygenated at various sites of the carbon skeleton. A few carbohydratebound compounds have also been encountered. Although several degraded carotenoids are present in green leaves of tobacco, the structural diversity is considerably greater and the concentration is much higher in cured (dried) leaves. Little is known about the cleavage reactions, which convert parent carotenoids into primary degradation products. Some insight, however, has been obtained by biomimetic syntheses. Possible pathways for the biogeneration of selected carotenoid-derived aroma compounds, which are based on results from such model experiments, are discussed.

Our present knowledge of degraded carotenoids in tobacco originates from studies initiated in the late 1960s. In these studies gas chromatography coupled to mass spectrometry was commonly used as the main tool, and the objective was to identify the aroma compounds of tobacco. The results revealed that the volatile aroma fractions derived from tobacco are complex mixtures composed not only of degraded carotenoids but also of other degraded isoprenoids, monoand sesquiterpenoids, fatty acid metabolites, phenylalanine metabolites, alkaloid (nicotine) metabolites and products of sugar-amino acid reactions. The

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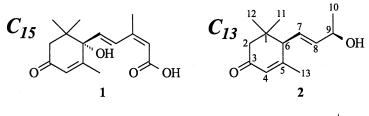
degradation products derived from carotenoids received particular attention because of their utmost importance to the aroma. These findings gave the incitement to further studies and as a result many new apocarotenoids were isolated and their structures and absolute configurations were elucidated. Biomimetic studies were performed in order to confirm the structural assignments and to obtain insight into the mode of formation of these compounds. Research in this area remained very active until the early 1990s (*1*-4); only a few articles on carotenoid-derived compounds in tobacco have been published since then (5,6).

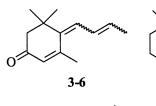
In this chapter the structural diversity encountered among the carotenoid derived compounds of tobacco is described and the possible mode of formation of selected key compounds is discussed.

Structural Diversity

More than one hundred carotenoid derived compounds have been identified in tobacco. These include apocarotenoids having 15, 13, 12, 11, 10 or 9 carbon atoms (Figure 1) as well as carbohydrate-bound apocarotenoids (Figure 2). (S)-Abscisic acid (ABA) (1), which is now recognized as a genuine C_{15} apocarotenoid, is a constituent of tobacco. The largest structural diversity is found among the C_{13} apocarotenoids. These count together more than sixty compounds and vary with respect to the degree and pattern of oxygenation and unsaturation. Most have been found in many other plant species but a few seem to be typical of tobacco. The C₁₃ apocarotenoids are represented in Figure 1 by (6R,7E,9R)-9-hydroxymegastigma-4,7-dien-3-one (3-oxo- α -ionol) (2), isomeric megastigma-4,6,8-trien-3-ones (3-6), 4-hydroxy- β -ionone (7), (5R,6S,7E,9S)megastigm-7-ene-5,6,9-triol (8), (3S,5R,6S,7E,9R)-5,6-epoxy-megastigm-7-ene-3,9-diol (9), damascenone (10), (3R)-3-hydroxy- β -damascone (11), (2R,8aR)-3,4-dihydro-3-oxoedulane (12) and the aspirone (13). C_{12} apocarotenoids, whose formation cannot be explained by simple cleavage of the polyene chain in a carotenoid precursor, are rare in nature. One compound, i.e. ketone 14, has been found in tobacco. The group of C_{11} apocarotenoids comprises more than ten compounds and is exemplified by dihydroactinidiolide (15) in Figure 1. The C_{10} and C_9 apocarotenoids are represented by β -cyclocitral (16) and oxoisophorone (17)(4).

In Figure 2 sugar-bound C_{15} (18), C_{13} (19-26) and C_{11} (27) apocarotenoids are gathered. Of these, the glucosyl ester of (S)-abscisic acid (18) has been isolated from tobacco stigmas (7). The sugar bound C_{13} apo-carotenoids have the glucose unit attached to the C-9, C-3 or both positions. These compounds and the glucoside of the C_{11} compound loliolide have been isolated from fluecured Virginia tobacco and/or sun-cured Greek tobacco (5,6,8-10).

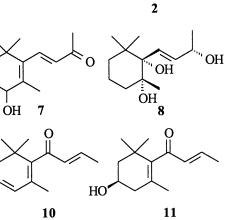


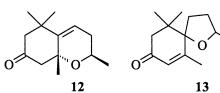


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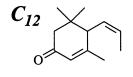
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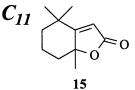




Figure 1. Examples of apocarotenoids in tobacco.

Glycosides have not, as yet, been isolated in pure form from air-cured burley tobacco, and the glycoside-containing fraction isolated from this tobacco is reported to be considerably smaller than that obtained from Virginia tobacco (11). From a biogenetic point of view it is of interest to note that the presence of glycosides has also been ascertained in green leaves of tobacco by the isolation of 3-oxo- α -ionol (2) and vomifoliol (28) after enzymatic hydrolysis of glycoside-containing fractions (12).

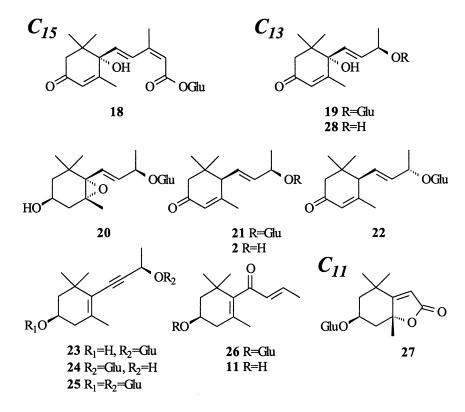


Figure 2. Sugar-bound apocarotenoids in tobacco.

The ratio bound to free form of apocarotenoids seems to vary with tobacco type, or, most likely the method of curing applied. Thus, Heckman and coworkers concluded that some of the glycosidically bound apocarotenoids in Virginia tobacco are present in levels that rival the levels of the corresponding free apocarotenoids (13). In Oriental tobacco, however, the free forms of (3R)-3-hydroxy- β -damascone (11) and 3-oxo- α -ionol (2) were reported to heavily outweigh the bound forms (6). It is noteworthy that in fruits and berries,

glycosides of apocarotenoids are often present in amounts exceeding those of the corresponding aglycones by a factor of 2 to 5 (14).

Carotenoids in Tobacco

The major carotenoids in green leaves of tobacco are the ubiquitous lutein, β , β -carotene, neoxanthin and violaxanthin. β , ϵ -Carotene, cryptoxanthin, zeaxanthin, flavoxanthin, antheraxanthin and other, as yet, unidentified carotenoids and *cis*-isomers of known carotenoids are present in smaller quantities. The absolute amounts and relative concentrations vary with factors such as tobacco type and position of the leaf on the plant (15).

Carotenoid Degradation and Generation of Apocarotenoids in Tobacco

It has been shown that the carotenoids undergo degradation both during growth and curing. Thus, burley tobacco is slowly degraded during senescence and air-curing. The level of lutein was found to decrease from 1.22 to 0.75 mg/g during the four weeks from topping of the tobacco plant to the time of harvest. A further decrease to 0.22 mg/g was observed during the 90 days of air-curing. β , β -Carotene was degraded to the same extent as lutein: from 0.69 to 0.12 mg/g. Violaxanthin and neoxanthin were metabolized more extensively and levels as low as 0.01 mg/g were found in the air-cured leaves (16). A degradation pattern similar to that of the carotenoids in burley tobacco was observed in Virginia tobacco (17).

The degradation of the carotenoids is accompanied by an increase in the level of apocarotenoids in processed tobacco. This was amply demonstrated in a study performed in the 1970s (18). Fractions obtained by headspace techniques from green, yellowed and flue-cured leaves of a Virginia tobacco, and from the flue-cured leaves aged for 6, 12 and 24 months were compared with respect to their content of volatile components. Nineteen degraded carotenoids were identified altogether. The majority of these were present in green leaves, but curing and aging generally resulted in an increase of the concentrations to the levels typically observed in processed tobacco, i.e. ng/g to $\mu g/g$ of tobacco.

The results obtained showed that the four geometrical isomers of megastigma-4,6,8-trien-3-one (3-6), β -damascone (29, Figure 3), 3-hydroxy- β -damascone (11), 3,4-dihydro-3-oxoedulane (12), and dihydroactinidiolide (15) increased in concentration during the post-harvest treatment. Damascenone (10), megastigma-4,7,9-trien-3-one (30) and 3,4,6,7-tetrahydro-3-oxoedulane (31) were generated *de novo* during the flue-curing or aging processes (18).

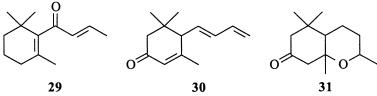


Figure 3. Formulae 29-31

Proposed Biogenesis – Primary Cleavage Products

It is evident from the study presented above that both the structural diversity and amounts of apocarotenoids in tobacco are highly affected by the post-harvest treatment. The results obtained cannot be used, however, to identify the primary degradation products, nor do they give a detailed insight into the subsequent metabolism of the primary products. Furthermore, since the enzyme systems that are responsible for the oxidative degradation of the tobacco carotenoids have not as yet been studied, the general model comprising dioxygenase-assisted cleavage of the 11,12, 9,10, 8,9, 7,8 and 6,7 bonds in a parent carotenoid with formation of C₁₅, C₁₃, C₁₁, C₁₀ and C₉ primary degradation products is adopted for tobacco in the discussion below. This model rests on results obtained from experiments involving auto-oxidation, photooxygenation, heat treatment and enzyme treatment of single carotenoids. Supporting evidence has also been provided by observations of identical stereochemistry/absolute configuration in the presumed carotenoid precursor and the degradation product. The isolation of compounds that have retained the cyclic end group as well as complementary in-chain compounds from saffron, quince and garden roses has also been cited as evidence in favor of this biogenetic model (19).

The carotenoids present in tobacco would hence give rise to the C_{13} apocarotenoids shown in Figure 4. It is interesting to note that four of these, β -ionone (**32**), α -ionone (**33**), (3*R*)-3-hydroxy- β -ionone (**34**), and (3*S*,5*R*,6*S*,7*E*)-3-hydroxy-5,6-epoxy- β -ionone (**35**) have been encountered in tobacco. The remaining three, i.e. the grasshopper ketone (**36**), derived from the end group in neoxanthin, (3*R*)-3-hydroxy- α -ionone (**37**), from the end groups of flavoxanthin and lutein, and (3*S*,5*R*,8*S*)-5,8-epoxy-3-hydroxymegastigm-6-en-9-one (**38**), from the end group of flavoxanthin, have not yet been discovered in tobacco. C_{13} Apocarotenoids, whose formation may be accounted for by subsequent metabolism of these three primary degradation products do, however, occur in tobacco.

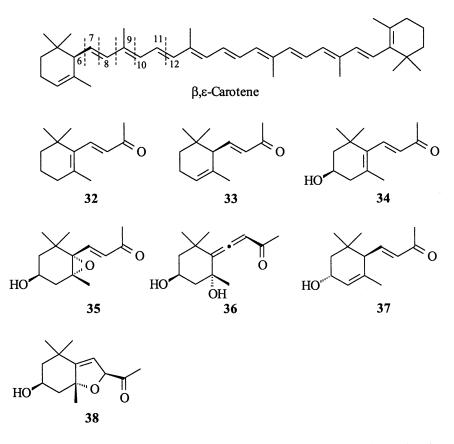


Figure 4. Proposed biogenesis of primary cleavage products and postulated primary cleavage products in tobacco.

Proposed Biogenesis - Products of Secondary Reactions

The primary degradation products are proposed to undergo further metabolism involving oxidation, reduction, dehydration and rearrangement. Glycosides may be formed and these generate volatile aglycones on acid- or enzyme-catalyzed hydrolysis or elimination. The discussion below includes proposed biogenesis of selected apocarotenoids that are important in tobacco.

Megastigmatrienones and Related Compounds

The isomeric megastigma-4,6,8-trien-3-ones (3-6) (20) are noteworthy because of their aroma properties; they have been referred to as the heart of tobacco aroma (21). They occur in tobacco along with several biogenetically closely related C_{13} compounds oxygenated at C-3. Some of these are found in the biogenetic scheme shown in Figure 5.

(3R)-3-Hydroxy- α -ionone (37) has been invoked as the precursor of these compounds. The proposed route to the megastigma-4,6,8-trien-3-ones (3-6) involves oxidation of 37 to give 3-oxo- α -ionone (39) followed by reduction to 3-oxo- α -ionol (2). The latter, or the corresponding glucoside (21) undergoes rearrangement with formation of 3-oxo-*retro*- α -ionol (40) and subsequent dehydration (4). Compound 40 is also a synthetically verified intermediate in the formation of the 3,4-dihydro-3-oxoedulanes 12 and 41. Since the latter two compounds have been assigned (2R,8aR)- and (2R,8aS)-configurations, 3-oxo-*retro*- α -ionol (40) should have a (9R)-configuration (22). The isomeric 3,4,6,7-tetrahydro-3-oxoedulanes 31 are suggested to arise by an analogous rearrangement of (6R,9R)-9-hydroxymegastigm-4-en-3-one (42) (4).

3-Oxo- α -ionol (2) is a well-known and fairly abundant tobacco apocarotenoid. It occurs as a mixture composed of the (6*R*,9*R*)- and (6*R*,9*S*)isomers as the main components and the corresponding enantiomers as the minor components (6, 23). The discovery of the (6*S*)-isomers is of interest, since it implies that epimerisation may take place at C-6 in 3-oxo- α -ionone (39) or that tobacco may contain (6*S*)-carotenoid precursors.

The presence of the (3S,6R,7E,9R)- and $(3S^*,6R^*,7E,9S^*)$ -megastigma-4,7diene-3,9-diols (43, 44) in tobacco is also noteworthy from a biogenetic point of view (23). These diols may arise via a stereospecific reduction of the 3-oxo group in 3-oxo- α -ionone (39) or by reduction of (3S)-3-hydroxy- α -ionol (45). The latter, in turn, would originate from 3'-epilutein. This carotenoid has not, however, been encountered in tobacco.

Damascenone and Related Compounds

Damascenone (10) and β -damascone (29) are highly valued aroma compounds. They have been found in many fruits, berries and other plant material. As early as 1971 both compounds were isolated from tobacco (24), where they cooccur with structurally closely related compounds, such as (3*R*)-3-hydroxy- β damascone (11) (25) and the bicyclodamascenones 46 and 47 (26).

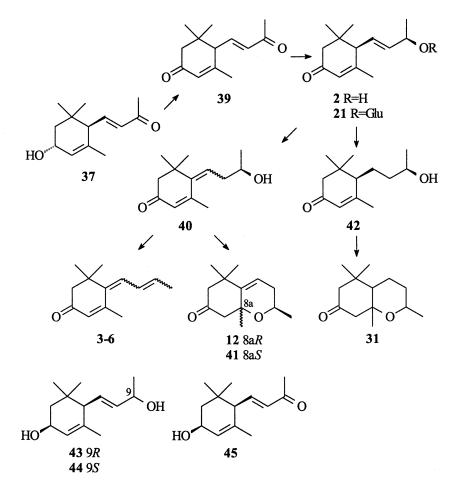


Figure 5. Proposed biogenesis of megastigma-4,6,8-trien-3-ones 3-6 and edulanes 12, 31, 41, and 42. Formulae 43-45.

Based on results from early model experiments with C_{13} precursors and from findings of more recent experiments with carbohydrate bound precursors, the formation of damascenone (10) and (3*R*)-3-hydroxy- β -damascone (11) may be rationalized as shown in Figure 6 (19). Grasshopper ketone (36) is reduced to triol 48. Subsequent rearrangement of triol 48 or of the corresponding 9-*O*- β -Dglucoside (49) results in transposition of the oxygen function from C-9 to C-7 with formation of damascenone (10) and (3*R*)-3-hydroxy- β -damascone (11). The acetylenic diol 50 may also be involved as an intermediate. Although the grasshopper ketone (36) has not, as yet, been detected in tobacco, support for

the view that this biogenetic scheme is valid also for tobacco comes from the fact that the acetylenic diol **50** as well as the corresponding glucosides (**23-25**) are tobacco constituents (5). In addition, the acetylenic diol (**50**), damascenone (**10**) and 3-hydroxy- β -damascone (**11**) have all been isolated as aglycones after treatment of glycoside-containing tobacco fractions with hydrolytic enzymes (6, 11,13). The bicyclodamascenones **46** and **47** have been isolated from flue-cured Virginia tobacco. They have been prepared synthetically by treatment of damascenone (**10**) with acid (26).

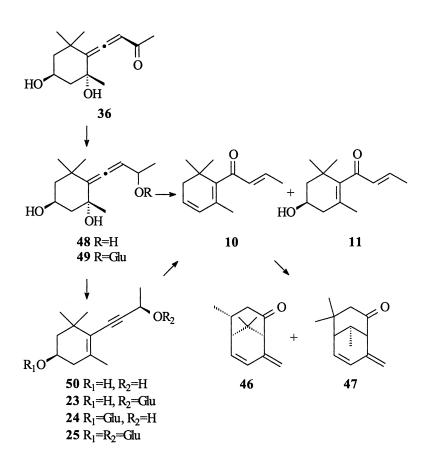


Figure 6. Proposed biogenesis of damascenone (10), 3-hydroxy- β -damascone (11) and the bicyclodamascenones 46 and 47.

The generation of β -damascone (29) has probably a different course and may be accounted for as outlined in Figure 7. β -Ionone (32) is reduced to β ionol (51). The latter undergoes an oxygenase-induced conversion into the allenic diol 52 followed by the required rearrangement into β -damascone (29). Consistent with this pathway is the finding that the allenic diol 52 is one of the products obtained from β -ionol (51) by sensitized photooxygenation followed by reduction of the hydroperoxides formed. It is worthy of mention that one of the other two ene-products (53, 54) of this reaction, megastigma-5(13),7-diene-6,9-diol (53), has also been isolated from tobacco (27).

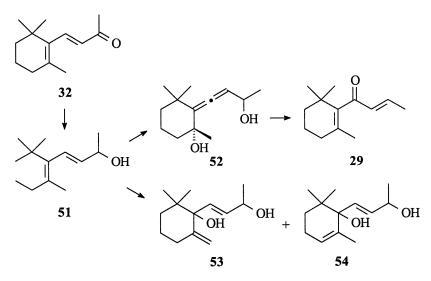


Figure 7. Proposed biogenesis of β-damascone (29) and megastigma-5(13),7diene-6,9-diol (53).

3,6-, 5,6- and 5,8-Epoxy-bridged Megastigmenes

 $(3S^*, 5R^*, 6R^*, 7E, 9R^*)$ -3,6-Epoxymegastigm-7-ene-5,9-diol (55) contains a 3,6-epoxy bridge, a structural feature that is unusual among the naturally occurring megastigmanes. This compound is reported to have a weak hay-like aroma (28-30).

Both the 3,6-epoxide 55 and (3S,5R,8S)-5,8-epoxy-6-megastigmene-3,9-diol (56), another tobacco component, have been obtained as products on treatment of (3S,5R,6S,7E)-5,6-epoxymegastigm-7-ene-3,9-diol (9) with weak

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acid (28). It seems likely therefore that their biogenesis takes place as illustrated in Figure 8 with the primary cleavage product 35 as the precursor.

The 5,8-epoxide 56, which readily undergoes oxidative degradation with formation of the C_{11} lactone (-)-(3S,5R)-loliolide (57) (28), may also derive from the 5,8-epoxy bridged C_{13} primary cleavage product (38) of flavoxanthin.

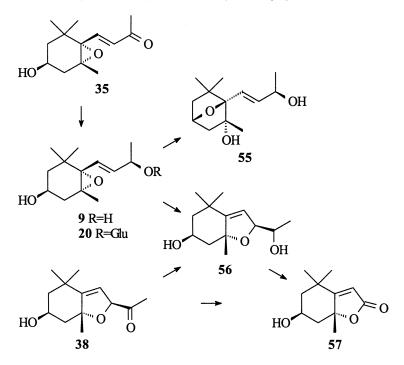


Figure 8. Proposed biogenesis of the 3,6-epoxide 55, the 5,8-epoxide 56 and (3S,5R)-loliolide (57).

Seco-apocarotenoids

The isolation of seco-compounds such as the C_{10} compound 3,3-dimethyl-7-hydroxyoctan-3-one (58) (31) indicates that the metabolism of the apocarotenoids is very extensive in tobacco. This is most likely explained by the post-harvest treatment.

The biogenesis of **58** may formally be envisaged to occur via cleavage of the 5,6-bond in the C_{10} triol **59** (Figure 9). This route could not, however, be

verified by chemical means, since attempted ring cleavages were unsuccessful. The desired cleavage was instead accomplished via oxidation of diol 60 (31).

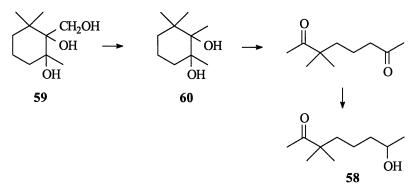


Figure 9. Proposed biogenesis of the seco-apocarotenoid 3,3-dimethyl-7hydroxyoctan-2-one (58).

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Chapter 11

Carotenoid-Derived Aroma Compounds in Tea

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The effects of the tea manufacturing process (heat-drying, pan-firing, solar-drying and fermentation) on the production of carotenoid-derived aroma compounds are discussed. Several carotenoid-derived aroma compounds are important in the development of fine tea flavor. In the present study, we analyzed the volatile components of tea infusions using a brewed extraction method and gas chromatography/mass spectrometry (GC/MS). An analysis of aroma patterns showed that the concentrates prepared by the brewed extraction method are quite different from those obtained by the steam distillation/extraction (SDE) method. Extracts of black tea that were prepared using the brewed extraction method contained only dihydroactinidiolide and theaspirone as carotenoid-derived aroma compounds. The photo-oxidation effect of solar-drying was also examined using a model experiment.

Young tea leaves from *Camellia sinensis*, called "tea flush", contain 6.48 to 21.66 mg % of β -carotene and 18.74 to 35.61 mg % of lutein and zeaxanthin in the dry weight (1). Tea products are also known to contain many carotenoid-derived aroma compounds (2). The loss of total carotenoids during the manufacturing process was reported to be as high as 38 to 44 % for green tea (3) and 20 % (1) to 40 % (4) for black tea. The tea manufacturing process of various teas is outlined in Scheme 1. None of carotenoid-derived aroma compounds were present in the genuine tea leaves (3). Heat treatment such as steaming, pan-

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firing, heat-rolling and drying, photo-treatment such as solar-withering and solar-drying, fermentation treatment using leaf enzymes such as withering and indoor-withering, and fermentation treatment using microbes such as in pickling and pilling seem to contribute to carotenoid degradation and the production of carotenoid-derived metabolites. Actually, in our studies, some carotenoid-derived aroma compounds were found in manufactured tea (5,6). Kabuse-cha, a green tea made from tea leaves that are grown in the shade, contains larger amounts of carotenoid-derived aroma compounds and even larger amounts of carotenoid-derived aroma compounds in comparison with normal green tea, such as *Sen-cha*. One factor contributing to the characteristic aroma of Kabuse-cha, called 'Ooika', may be the carotenoid-derived aroma compounds (3).

tea leaf → steaming (*H*) →rolling →heating rolling (*H*) →drying (*H*) →refining (*H*) →Green tea (Sen-cha, Kabuse-cha, Gyokuro)

low material crude green tea including tea shoots →roasting (*H*) →Hoji-cha

tea leaf → pan-firing (*H*) → rolling → pan-firing (*H*) → drying (*H*) → refining (*H*) → Green tea (*Kamairi-cha, Pan-fired tea*)

tea leaf → solar-withering (S) → indoor-withering (E) → panning (H) → rolling \rightarrow drying (H) → **Oolong tea**

<u>tea leaf</u> → withering (E) → rolling (E) → fermenting (E) → drying (H) → Black tea

tea leaf → steaming (H) → pickling (microbial fermenting) → solar-withering (S) → Microbial fermented tea (Goishi-cha, Awa-cha)

tea leaf → steaming (*H*) → rolling → piling (*M*) → solar-drying (*S*) → Microbial fermented tea (*Toyama-kurocha*)

<u>tea leaf</u> → pan-firing (*H*) → rolling → solar-drying (*S*) → piling (*M*) → solardrying (*S*) → piling (*M*) → **Microbial fermented tea** (*Puer-tea*)

Scheme 1. Tea Manufacturing process. (H: heat treatment, S: solar-treatment, E: leaf enzyme fermentation, M: microbial fermentation).

Analysis of Carotenoid-derived Aroma Compounds

We have determined that several previously identified aroma compounds were actually thermal degradation products resulting from sample preparation methods such as SDE (using a modified Likens-Nickerson's apparatus) and SDR (Steam Distillation under Reduced Pressure using rotary evaporator). The volatile components of tea infusions prepared using a brewed extraction method were analyzed by gas chromatography/mass spectrometry (GC/MS). Based on our results (7, 8), this method substantially reduces sample degradation prior to GC/MS analysis. Aroma pattern analysis shows that the concentrates prepared by the brewed extraction method are quite different from those obtained by the SDE method as shown in Table I (8,9,10) and Figure 1 (10).

The yield of aroma compounds derived from carotenoids was very low. This was especially true for the brewed extract, or tea infusions. The pan-fired green tea brewed extract contained a high amount of dihydroactinidiolide. The amount of theaspirone, the most important tea aroma component, was 1.3 % of the total aroma content. The amounts of 2,6,6-trimethyl-2-hydroxycyclohexanone, 3,3-dimethyl-2,7-octanedione and 5,6-epoxy- β -ionone were as high as 1 % of the total aroma content. The brewed extract contained lower amounts of β -ionone than the SDE extract.

The brewed extract of black tea only contained dihydroactinidiolide and the aspirone as carotenoid-derived aroma compounds. However, the SDE extract contained additional compounds such as 5,6-epoxy- β -ionone, safranal, β -ionone, nerolidol and β -cyclocitral, which are obviously thermal degradation products formed during the SDE process.

Oolong, a semi-fermented tea, contained additional carotenoid-derived aroma compounds. The extract of oolong tea using the SDE method showed nerolidol and α -farnesene as the major components (11,12). When aroma patterns from the brewed extract and the SDE extract are compared, however, nerolidol and α -farnesene appear to be thermal degradation products that are formed during the SDE process, as shown in Figure 1. The precursors of terpene alcohols have been reported to be glycosidic derivatives such as primeverosides (13,14,15), but in these analyses no glucoconjugate of nerolidol has been detected. Therefore, it is likely that the precursor of nerolidol and α -farnesene is the carotenoid phytofluene. A possible mechanism of formation of nerolidol and α farnesene is through epoxidation and dehydration of phytofluene (cf. Figure 2).

In the brewed extract of Puer tea, dihydroactinidiolide, a degradation product of β -carotene, was a significant component of the tea aroma. Carotenoid-derived aroma compounds comprised 14.2 % of the total aroma components for the Puer tea. These results indicate that degradation and oxidation occur strongly in the Puer tea manufacturing process.

While some of the carotenoid-derived aroma compounds have an attractive odor, excessive oxidation degrades the odor quality. Teas that have a fresh odor, such as green tea, oolong tea and black tea, generally contain low amount of degradation compounds.

	Gree	n tea	Oolor	g tea	Blaci	k tea	Pue	r tea
Compound (peak area %)	SDE ¹	BE ²	SDE	BE	SDE	BE	SDE	BE
2,6,6-Trimethyl-2-cyclohexanone	-	-	-	-	-	-	0.2	trace
6-Methyl-5-hepten-2-one	-	-	0.6	0.3	0.1	-	0.2	0.2
6-Methyl-(E)-3,5-heptadien-2-one	0.1	0.1	0.2	-	-	-	0.1	0.5
2,6,6-Trimethyl-2-hydroxycyclo- hexanone	1.9	0.7	-	0.2	0.2	-	0.8	0.6
β-Cyclocitral	1.1	-	0.3	-	0.3	-	0.4	-
Safranal	0.6	-	0.4	-	1.0	-	0.3	-
2,6,6-Trimethylcyclohex-2-en-1,4- dione	0.2	0.1	0.5	-	-	-	0.1	0.5
α -Farnesene	-	-	2.6	0.3	-	-	-	-
α-Damascone	-	-	0.4	-	-	-	-	-
(E)-β-Damascenone	-	-	0.1	-	-	-	0.1	-
α-Ionone	-	-	0.6	-	-	-	0.5	-
Geranylacetone	0.2	-	0.6	-	0.2	-	0.7	-
3,3-Dimethyl-2,7-octanedione	-	0.4	-	0.2	-	-	-	0.2
β-Ionone	3.5	0.1	1.1	-	0.7	-	1.6	-
5,6-Epoxy-ß-ionone	2.2	0.6	0.6	0.3	0.1	-	0.9	0.5
Nerolidol	2.3	-	38.1	0.7	2.2	-	6.6	-
6,10,14-Trimethylpentadecanone	-	-	-	-	-	-	2.0	-
Theaspirone	0.5	1.3	0.3	0.8	-	0.6	-	1.4
4-Oxo-β-ionone	-	-	-	-	-	-	0.3	-
Dihydroactinidiolide	1.7	6.8	0.2	1.6	0.7	3.8	1.0	10.3
Total	14.3	9.9	46.5	4.3	5.5	4.3	15.8	14.2

Table 1. Carotenoid-derived Aroma Compounds in Tea

Green tea: Longjing tea, Oolong tea: Huang Chin Kuei, black tea: clone-2025 (8-10). Abbrev.: ¹Simultaneous distillation/extraction; ²brewed extract

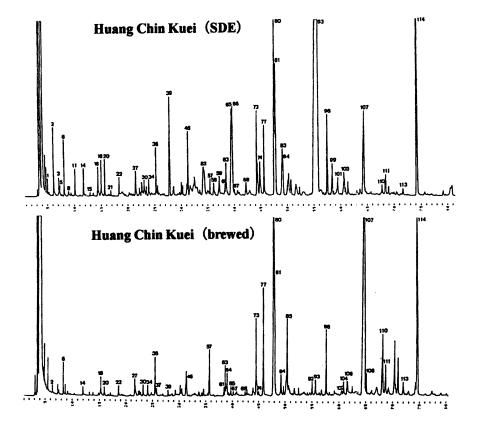


Figure 1. Gas chromatograms of Huang Chin Kuei oolong tea: brewed extract and SDE extract, respectively. PEG 20M 0.25 mm i.d. \times 50 m capillary column; $60^{\circ}C \rightarrow 2^{\circ}C$ /min \rightarrow 180°C. Peak 66: α -farnesene, peak 93: nerolidol, peak 110: dihydroactinidiolide

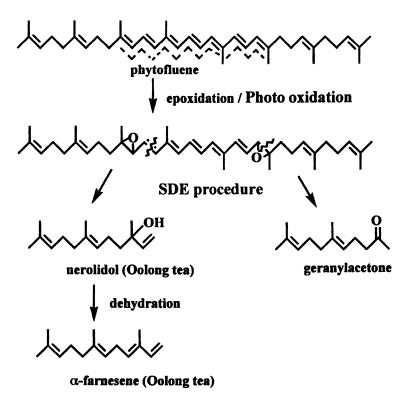


Figure 2. Proposed formation of nerolidol and α -farnesene from phytofluene.

Compound (mg/100 g dry weight)	steamed	fermented	dried	stored
6-Methyl-2-heptanone		-	_	0.1
6-Methyl-5-hepten-2-one	tr.	0.2	1	1.4
2-Methyl-2-hepten-4-one	-	tr.	0.1	0.1
2,6,6-Trimethyl-2-cyclohexanone	-	tr.	0.1	0.1
6-Methyl-(Z)-3,5-heptadien-2-one	-	tr.	tr.	tr.
6-Methyl-(E)-3,5-heptadien-2-one	-	-	0.3	1.5
2,6,6-Trimethyl-2-hydroxycyclo- hexanone	-	0.2	0.7	1.4
β-Cyclocitral	-	0.2	0.7	0.4
Safranal	-	-	0.1	0.2
2,6,6-Trimethylcyclohex-2-en-1,4-dione	-	1.1	0.9	1
6,10-Dimethylundecanone	tr.	0.1	0.6	1.8
α-Farnesene	0.8	0.7	-	tr.
α-Ionone	-	0.6	1.4	1.6
Geranylacetone	0.1	0.7	1.4	2.8
β-Ionone	-	0.8	1.3	1.6
5,6-Epoxy-ß-ionone	tr.	0.3	0.8	1.6
Nerolidol	0.8	3.3	2.3	2.3
6,10-Dimethyl-3,5,9-undecatrien-2-one	-	0.3	0.5	0.2
6,10,14-Trimethylpentadecanone	0.2	0.7	1.6	3.6
Theaspirone	-	0.1	0.1	0.1
Dihydroactinidiolide	-	0.3	3	0.8
Total	1.9	9.6	16.9	22.6
Total Carotenoids / Total Aroma	19.0 %	17.5 %	18.2 %	18.4 %

Table II. Changes in Carotenoid-derived Aroma Compounds in *Toyama*kurocha Manufacturing (SDE extract) (16).

Changes in Aroma Compounds during Manufacturing

The changes of the levels of carotenoid-derived aroma compounds in *Toyama-kurocha*, a Japanese microbial fermented tea, are shown in Table II.

The SDE extracts of *Toyama-kurocha* were prepared for the four different steps of the manufacturing process, i.e. steaming, fermenting, solar-drying and storing, and the results were compared (16). The composition and amount of carotenoid-derived aroma compounds at each manufacturing stage are extremely varied. Since this is attributed to the SDE extract, some compounds may be present as precursors in the tea. The steamed sample contained a small amount of carotenoid-derived aroma compound. After natural fungal fermentation for 20-25 days, numerous carotenoid-derived aroma compounds were produced. Solar-drying for 2-3 days increased photo-oxidative carotenoid degradation

products such as 2,6,6-trimethyl-2-hydroxycyclohexanone, β -cyclocitral, α ionone, 5,6-epoxy- β -ionone and dihydroactinidiolide. In a sample that was stored for 1 year, the amounts of 6-methyl-5-hepten-2-one, 6-methyl-(*E*)-5hepten-2-one, 6,10-dimethylundecanone and 6,10,14-trimethyl-pentadecanone were increased considerably. The pickling fermentation by lactobacilli did not result in carotene degradation that was observed in Japanese Awa-cha and Thailand Miang (17,18).

The SDE extracts prepared for the four different steps of the oolong tea manufacturing process, i.e. solar-withering, indoor-withering, mass-rolling and parching, showed that indoor-withering increased amounts of α -farnesene and nerolidol remarkably (11,12). However, the brewed extract of oolong tea contained a very small amount of these two compounds. It is presumed that the amount of the precursor compound to α -farnesene and nerolidol increases substantially under the action of oxidative enzymes that are present in the indoor-withering and turn over treatments. Nobumoto et al. reported that α farnesene was produced from the precursor nerolidol through a mechanism of spontaneous biological dehydration instead of the SDE process (19). It is also possible that a precursor is present during the enzymatic fermentation that is degraded to α -farnesene via nerolidol under conditions of the SDE process.

Effects of Heat Treatment

The effects of steam-heating and pan-firing during the tea manufacturing process on the production of carotenoid-derived aroma compounds from ßcarotene were examined using two model experiments. In the first experiment incorporating the pan-firing model, ß-carotene was heated at 180°C for 6 minutes. Ten volatile compounds were produced as shown in Table III (20). In a second experiment (steam-heating model) β -carotene was heated in a water bath at 90°C, 120°C and 150°C, respectively, for 90 minutes. Approxi-mately 40 degradation compounds were produced during this treatment. The principle carotenoid-derived aroma compounds are shown in Table IV (21).Dihydroactinidiolide, 5,6-epoxy-B-ionone, 3,3-dimethyl-2,7-octanedione, and 2,6,6-trimethyl-2-hydroxycyclohexanone are present in large amounts. Only these four compounds were found in Kamairi-cha brewed extract (9). The results of these two experiments seem to indicate that water, which is present during steam-heating, significantly affects the progress of oxidation. Since the level of β -ionone was not so high, steam-heating obviously does not result in a substantial oxidation of β -carotene.

Effects of Photo-oxidation

The effect of photo-oxidation on β -carotene during the oolong tea and Japanese microbial fermented tea process was examined using a simple model system.

Compound	(amount by rel. peak area)
Toluene	+++++++
Xylene	++
β-Cyclocitral	+++
Ionene	+++
3,3-Dimethyl-2,7-octanedione	++
Geranylacetone	+
β-Ionone	+++++++
5,6-Epoxy-β-ionone	+++++++
Dihydroactinidiolide	++++
Alkylbenzene	+++

Table III. Thermal Degradation Products of B-Carotene

Table IV. Thermal Degradation Products of B-Carotene (Steam Model)

Compound	90°C ^{1,2}	120°C ^{1,3}	150°C ^{1,4}
2,6,6-Trimethylcyclohexanone	0.2	0.1	0.5
2-Methyl-2-hepten-4-one	0.2	0.2	0.5
2,6,6-Trimethylcyclohex-2-enone	0.4	0.1	0.5
2,6,6-Trimethyl-2-hydroxycyclohexanone	3.8	3.5	9.2
β-Cyclocitral	0.8	2.9	1.0
Ionene	0.2	-	0.5
3,3-Dimethyl-2,7-octanedione	4.6	2.2	14.9
β-Ionone	2.5	7.9	1.5
5,6-Epoxy-β-ionone	24.3	17.1	9.0
2,6,6-Trimethyl-2,3-epoxy-cyclohexylidene-1- acetaldehyde	2.5	2.0	2.0
β-Damascone	0.2	-	0.2
Dihydroactinidiolide	35.5	42.2	45.4
4-Oxo-β-ionone	1.4	1.5	0.2

¹Peak area in %, ²Flowery sweet aroma; ³flowery, green, bitter; ⁴phenolic bitter

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In order to compare photo-oxidation in the two different processes, flasks of pure β -carotene and β -carotene contacted with water were subjected to 24 hours of sunlight. A blank sample was kept in the dark for a similar period of time. After exposure to sunlight, the flasks containing β -carotene were shaken to mix the volatile components into the water solution. The water solutions were filtered and the filtrates were then extracted with dichloromethane following the brewed extraction method. The results of GC-MS analysis of condensed dichloromethane solutions are shown in Figure 3 and Figure 4.

The predominant β -carotene photo-degradation products were dihydroactinidiolide, 3,3-dimethyl-2,7-octanedione, 5,6-epoxy- β -ionone and 2,6,6trimethyl-2-hydroxycyclohexanone. This result indicates that the photooxidation of β -carotene begins with epoxidation of the trimethylcyclohexenyl double bond which has steric hindrance, followed by additional epoxidation of other double bonds and by cleavage reaction of the epoxides. A proposed mechanism of formation for these β -carotene degradation compounds via photooxidation is shown in Figure 5. Our results also show that β -ionone is a rather minor photo-oxidation compound. Water also significantly affects the progress of the photo-oxidative reaction.

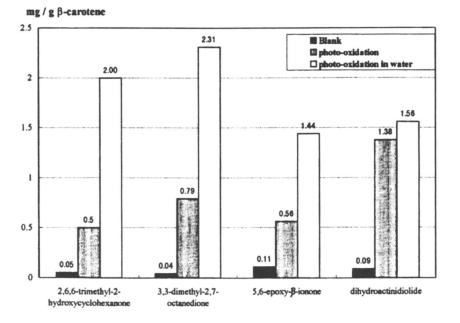


Figure 3. Predominant photo-degradation products from β -carotene.

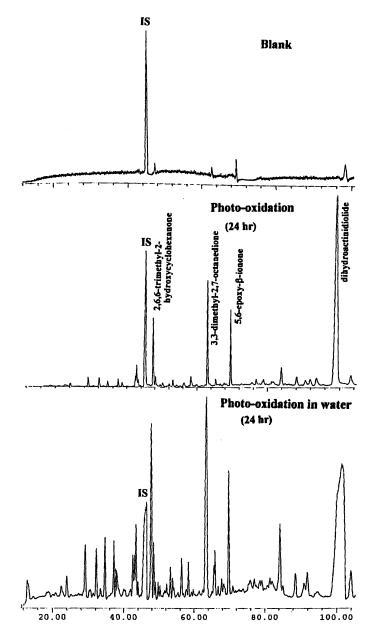


Figure 4. Gas chromatograms of photo-degradation products from β -carotene. HP-wax 0.25 mm i.d. ×60 m capillary column; 60°C \rightarrow 2°C/min \rightarrow 190°C.

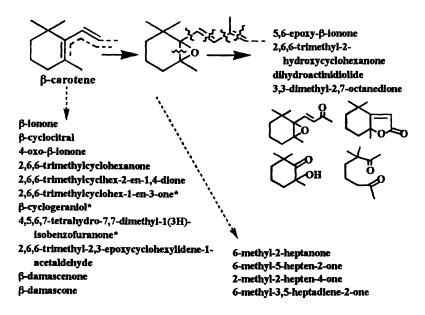


Figure 5. Proposed formation mechanisms of β -carotene-derived aroma compounds.

Effects of Enzymatic Oxidation

The manufacturing process for black tea and oolong tea involves naturally occurring enzymes in the tea leaves. Carotenoid degradation during the manufacturing process of black tea has been reported by Tirimanna and Wickremasinghe, who observed that several carotenoids disappeared during fermentation (22). Sanderson et al. (4) reported that fresh tea leaves contained 102 μ g of β -carotene, 260 μ g of lutein, 120 μ g of violaxanthin and 51 μ g of neoxanthin per g (dry weight). The authors also reported that these carotenoids decreased by 40 to 60 % during the first hour of fermentation. Mechanisms of enzymatic carotenoid degradation were studied using a model tea fermentation system composed of a soluble tea enzyme preparation, i.e. epigallocatechin gallate and β -carotene in a buffer system at pH 5.4 (4,23). The formation of β -ionone as the primary oxidation product of this system required both fermentation and a heat-drying process. Without both ingredients included,

neither fermentation nor heat drying processes produce β -ionone. Based on this result, Sanderson suggested a relationship between flavanol oxidation and oxidative degradation of carotenoid during the tea manufacturing as shown in Figure 6 (23).

The oxidation of carotenoid by a lipoxygenase system was also reported by various authors (24,25). Linoleate peroxy radical produced by spinach leaf lipoxygenase was reported to interact with lutein to produce the lutein bleaching product as shown in Figure 6 (26).

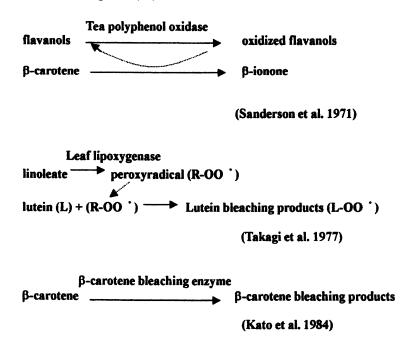


Figure 6. Enzymatic degradation mechanisms of carotenoids according to Refs. (4), (26), and (27).

A β -carotene bleaching enzyme has also been found in tea leaves (27). The enzyme had an optimum pH of 5.5-5.8, optimum temperature of 35°C and *Km* value of 5.56×10^{-6} mM for β -carotene. The authors suggested that a bleaching enzyme may be responsible for the degradation of β -carotene through β -apo-8'-carotenal as an intermediate.

Minor carotene degradation products are shown in Figure 7. Theaspirone seems to be mainly produced from lutein. Since theaspirone has only been found in teas that undergo the fermentation manufacturing process, it is considered to

be produced by enzymatic reaction. Interestingly, theaspirone has been identified in *Longjing* tea which is a green tea, and is most likely produced by the enzymatic action of the leaves during the characteristic mild pan-firing process.

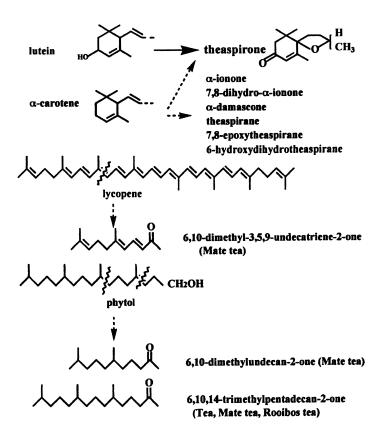


Figure 7. Minor carotene degradation products in tea.

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Chapter 12

Carotenoid-Derived Aroma Compounds in Flower Scents

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Flower containing carotenoid-derived scents aroma compounds have found an enormous appreciation during the past two decades in perfumery. Many of the famous modern fragrances contain high amounts of β-ionone, dihydro-βionone and related compounds, often in similar ratios as found in nature. Not surprisingly, 'ionone-floral' has even become a term in the verbal description of natural scents as well as fragrances. As might be expected, such flowers also contain the original carotenoids giving their coloring often between orange-yellow and yellow-brown, which, however, are assumed catabolize the of the to only in presence "cleaving enzymes". corresponding The present paper illustrates with the exemplary floral scents of Osmanthus fragrans, Boronia megastigma, Michelia champaca, various rose species and hybrides, various orchid species and some additional species of flowering plants the broad spectrum of natural products which may be generated by carotenoid catabolism in flowers.

Carotenoids are not only responsible for the colors of many plants, fruits, flowers, birds, insects and marine animals, they not only serve in combination

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with proteins as "Light Harvesting Complexes" in photosynthesis, and they are not only important in human nutrition as a source of Vitamin A and as a protection agent for cancer and heart disease – they are also the precursors of a vast variety of chemicals responsible for the fragrance of flowers and the flavor of foods as highlighted by this symposium.

Thus, flower scents containing carotenoid-derived compounds have found an enormous appreciation in perfumery during the past two decades. Many of the famous modern fragrances contain high amounts of β -ionone, dihydro- β ionone and related compounds, often in similar ratios as found in nature. Not surprisingly, "ionone-floral" has even become a term in the verbal description of natural scents as well as fragrances (1). This is due to their attractive olfactory profile, their potential to form new fragrance accords with other products, often combined with low odor thresholds. In flower scents they may occur in the extreme broad range of concentration between 95 % of all volatiles and trace constituents, still being of olfactory significance in the latter. Among the 1250 species of scented flowers investigated during the past 25 years in our laboratory, β -ionone occurs in 16 %, dihydro- β -ionone in 11 % and α -ionone in 4 % of all cases.

Despite considerable efforts in the understanding of the formation of these compounds, still little is known about the mechanism of carotenoid biodegradation. The *in-vivo* cleavage of the carotenoid chain is generally considered to be catalyzed by dioxygenase systems with a preference for the cleavage of the 9,10 (9*,10*) double bond as discussed by Winterhalter (2, and literature cited) and Eugster & Märki-Fischer (3) (cf. Fig. 1).

Table I gives an overview on the most common carotenoid-derived compounds and their occurrences in flower scents.

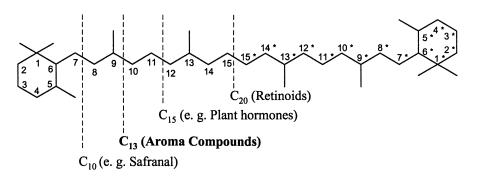
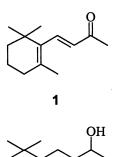
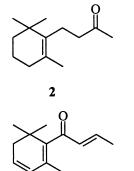


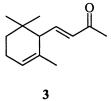
Figure 1. Major fragment classes of carotenoid biodegradation assumed to be formed by regioselective attack of dioxygenases as published by Ref. (2).

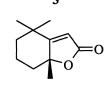
Name	Occurrence
β -Ionone (1) and	
Dihydro-β-ionone (2)	Viola odorata (4-6), Rosa species and hybrides (7-11), Osmanthus fragrans (12-15), Boronia mega- stigma (16-18), Michelia champaca (19), Gladiolus species (20), Telosma cordata (21), Lawsonia iner- mis (22), Freesia hybrida (23-24), vast variety of orchid species (1), Tulipa species and hybrides (26)
α-Ionone (3)	Viola odorata (4-6), Osmanthus fragrans (12-15), Acacia farnesiana (25), Boronia megastigma (16-18), Michelia champaca (19), Tulipa sylvestris (26), Aeschynomene fluitans (20), Orchid species (1)
Dihydro-β-ionol (4)	Osmanthus fragrans(13-15), Rosa species and hybrides (9,11), Michelia champaca (19), Rosa damascena (27)
(E)- β -Damascenone (5)	Osmanthus fragrans (13, 23), Primula secundiflora (20), Sambucus niger (20), Rosa damascena (27)
Dihydroactinidiolide (6)	



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Carotenoid-derived Compounds in Osmanthus fragrans

Among the 1250 species of flowering plants investigated by us those of the various varieties of *Osmanthus fragrans* show by far the highest diversity of carotenoid-derived constituents. This Chinese Oleaceae has already been lauded by Confucius and is still the Chinese standard for a beautiful flower scent. While the flowers of *Osmanthus* range from silver-white (*Osmanthus fragrans* Lour. var. *latifolius* Mak.) over gold-orange (*O. fragrans* Lour. var. *thunbergii* Mak.) to reddish (*O. fragrans* Lour. var. *aurantiacus* Mak.), the precious commercial extract (~US\$ 4000 per kilogram) is usually prepared from the gold-orange flowers, which tend to have more of the desirous notes connected to carotenoid-derived compounds. In fact, in such an *Osmanthus* absolute we could identify nearly 100 representatives of this group of natural products (*13*), β -ionone (1, 7.6 %), dihydro- β -ionone (2, 6.4 %), dihydro- β -ionol (4, 3 %), cisand trans-theaspirane (**33a/b**, 0.7 %) and α -ionone (**3**, 0.6 %) representing main components.

The three bicyclic oxa-compounds 9, 10 and 12 (13, 28-29) which have not yet been found in any other natural scents deserve first attention (cf. Figure 2). They may be derived from a carotenoid hydroxylated at carbon two and have the common intermediate (S)-2-hydroxy- β -ionone, which was also found as minor compound in *Osmanthus*. Mori and Tamura (30) have shown in the meantime by synthesis starting from (S)-3-hydroxy-2,2-dimethylcyclohexanone that the (E,E)-2,5-epoxy-6,8-megastigmadiene (9) isolated from Osmanthus is only of low optical purity being enriched to ca. 11 % e. e. in the (2S,5R)-(+)-isomer. They essentially followed the route applied by us (28) by conversion of (S)-7 with isopropenyl acetate and LAH reduction to the diol 8 and subsequent acid catalyzed cyclization to a 5 : 1 mixture of (2S,5R) -9 and (2S,5R) -10.

Also for the synthesis of the (2S,6R,7S)-2,7-epoxy-4,8-megastigmadiene (12) they followed the original route (29) and treated (S)-7 with NaOEt-EtOH to subsequently subject the oxa-ketone 11 to reduction, acetylation and pyrolysis. Percentage values in parenthesis in Figure 2 as well as in the following Figures refer to the content of the compound in the respective scent samples.

The 2,5-epoxy-6,8-megastigmadiene 9 is characterized by a refreshing odor reminiscent of cassis buds, exotic fruits and tomato leaves while the 2,7-epoxy-4,8-megastigmadiene 12 exhibits notes in the direction of rose, wine and red berries.

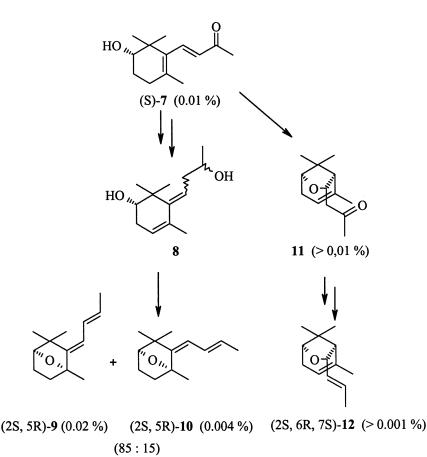


Figure 2. Bicyclic oxa-compounds unique to Osmanthus fragrans (28).

Acid-catalyzed cyclization of the diol 8 led not only to the target compounds 9 and 10 but also to the less volatile products 13 - 16 (cf. Figure 3). Since 13 a/b - 15 also occur as trace constituents in the investigated *Osmanthus* absolute (13) it may be assumed that the diol 8 is also of importance in the biological formation of 9 - 15.

A second synthesis for **9** and **10** published in 1989 by Di Fazio et al (31) uses the Diels-Alder reaction of 2-methylfuran with 2-chloroacrylonitrile to get the key intermediate 1,3,3-trimethyl-7-oxabicyclo [2.2.1] heptan-2-one.

Besides these unique bicyclic oxa-compounds also a series of unsaturated oxomegastigmanes are of structural and olfactory interest to Osmanthus (cf. Figure 4). Thus, the identification of the (E)-5,7,9-megastigmatrien-4-one (17) (13, 32) filled a gap in the chemistry of carotenoid-derived compounds. It bears

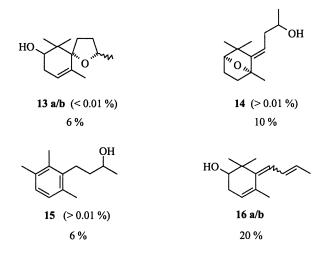


Figure 3. Further Osmanthus constituents derived from diol 8.

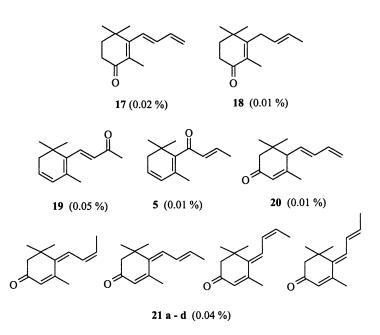


Figure 4. Unsaturated oxomegastigmanes in Osmanthus fragrans (32).

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the same oxidation state as the dehydro- β -ionone (19), the damascenone (5) being famous for its identification in *Rosa damascena* (27), the (E)-4,7,9-megastigmatrien-3-one (20) and the well known tobacco constituents 21 a-d (33), all of which are present in the Osmanthus absolute.

The trienone 17 is accompanied by the (E)-5,8-megastigmadien-4-one (18) which was identified at around the same time also in passion fruit and Virginia tobacco (34).

Both unsaturated oxomegastigmanes 17 and 18 have been synthesized starting from α -ionone epoxide 22 (13, 32). Treatment of 22 with catalytic amounts of sodium methylate in methanol (60 °C, 4 h) led to the 4-hydroxy- β -ionone (23) which was converted by oxidation and subsequent selective reduction and dehydration to 17. Interestingly, treatment of 22 with molar amounts of sodium methylate in methanol (60 °C, 6 h) gave directly the 4-oxo-dihydro- β -ionone (26) which was then subjected to the same reaction sequence to give 18. As indicated by the percentages in parenthesis all bifunctional carotenoid-derived compounds in Figure 5 could also be identified in the investigated absolute.

 α -Ionone epoxide (22) was found to be also the starting material for a fourth bicyclic oxa-compound unique to Osmanthus fragrans, the (E)-4,7-epoxy-5(11),8-megastigmadiene (29) (13). This trace constituent shows a refreshing fruity, floral odor reminiscent of the top note of geranium oil. It was obtained as the main compound by treating 22 with aluminium isopropylate in isopropanol (130 °C, 1.5 h).

An impressive group of Osmanthus constituents has been found to be derived from (+)-theaspirane A (33 a) and (+)-theaspirane B (33 b) (13, 35). As illustrated by Figure 6 the (E)- and (Z)-retroionols (32 a/b) might be of central importance for their biological formation. They could be formed by photoisomerization of α -ionone (3) and subsequent reduction. The isolated (+)theaspirane A (33 a) has been directly related to the known (-)-(2S, 5S)the aspirone (34 a) (35) whose absolute stereochemistry had been elucidated by Weiss, Galbraith et al (36, 37). The same relationship has been established for (+)-theaspirane B, (33 b) and 34 b. Both the theaspirones (34 a/b) are also present in the Osmanthus absolute in trace amounts, but more important in view of their odor contribution have been found to be the 7-oxo-dihydrotheaspiranes (35 a - 35 d) which show woody, cedar-like notes accompanied by aspects of dried fruits and patchouli leaves. Their synthesis from natural 33 a and 33 b clearly assign the (2S, 5S)-configuration to 35 a and 35 b and the (2R, 5S)configuration to 35 c and 35 d. As described by us (35) the investigated Osmanthus absolute contains also the epoxytheaspiranes and some isomers of 6hydroxy- and 7-hydroxy-dihydrotheaspirane as well as a series of further carotenoid derived compounds (13).

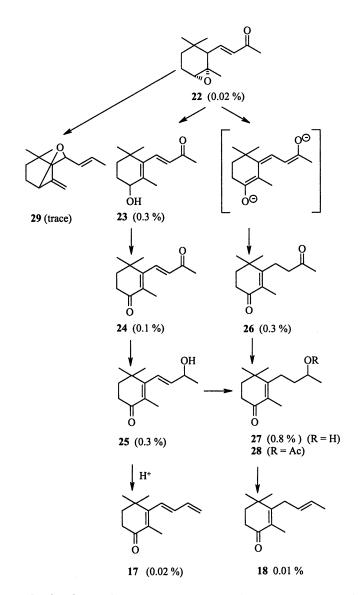


Figure 5. Synthesis of 17, 18 and 29 starting from α -ionone epoxide 22. Compounds 22-27 are also found in Osmanthus absolute.

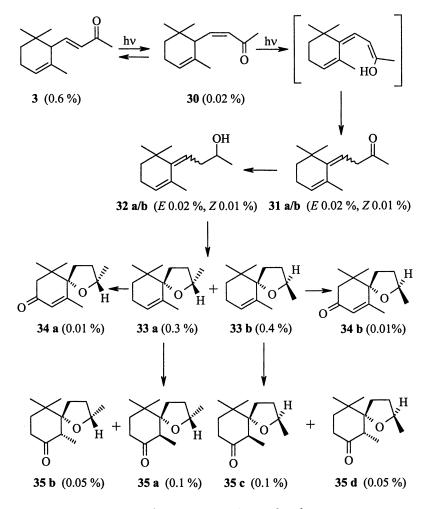


Figure 6. Theaspiranes in Osmanthus fragrans.

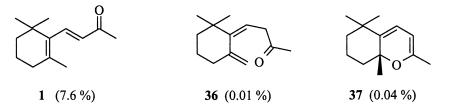


Figure 7. Photoisomers of β -ionone occurring in flower scents.

Also the main constituent β -ionone (1) seems to be partially transformed in the flower matrix by photoisomerization (cf. Figure 7). Thus, it is accompanied by its photocyclization product **37**, the so-called cyclic β -ionone, and the corresponding intermediate (Z)-retro- γ -ionone (**36**) (*38*).

We have found the combination of 1 and 37, mostly accompanied by 36, in the β -ionone-rich floral scents of many additional species as in those of *Viola* odorata (20), Encyclia adenocarpa (1), an epiphytic orchid native of Guatemala, Maxillaria nigrescens (1), a mysterious-looking epiphytic orchid growing in poorly accessible precipies of the Colombian and Venezuelan Andes, Oncidium tigrinum, a Mexican epiphytic mountain orchid providing an unforgettable olfactory experience, Gladiolus carinatus (20), the ionone-richest of the many Gladiolus species native of South Africa, and Lecythis sp. FG 44/1 (20, 39), one of the many Lecythidaceae native of Lower Amazonia and emitting ionone-floral scents. As summarized by us (13) the investigated Osmanthus absolute contains also a series of carotenoid-derived compounds with 11, 10 and 9 carbon atoms.

Carotenoid-derived Compounds in Boronia megastigma

Another scent extremely rich in carotenoid-derived compounds, especially β -ionone (1), is emitted from the cup-shaped flowers of *Boronia megastigma* Nees (Rutaceae), a protected species native of Southwestern Australia. Menary (*16*) was the first to publish a more detailed analysis of some *Boronia* concretes produced from selected clones cultivated in Southern Tasmania. Today, Boronia absolutes originating from this region and exhibiting a tremendously rich odor are commercially available. Their high prices allow, however, to use them only in the most precious fragrances and flavors. Our investigation of such an absolute (*17*) allowed us to identify among others some additonal carotenoid-derived compounds as the theaspiranes (**33a/b**), the cyclic β -ionone (**37**), β -ionol, the analogues of the α -series, all also present in the already discussed absolute of *Osmanthus fragrans*. Of special structural interest is the 7,11-epoxy-

5(6)-megastigmen-9-one (38), known as minor constituent of passionfruit (40) and identified by us in a series of orchid scents as main constituent (1, 53) and the 5,8-epidioxy-6-megastigmen-9-ol (39) which might have been formed via a 1,4-addition of oxygen to β -ionol.

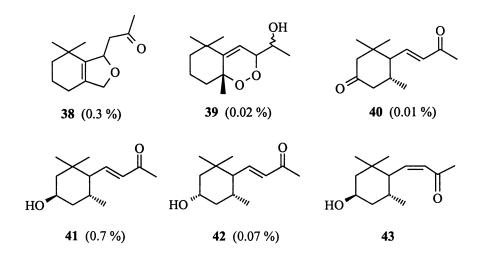


Figure 8. Bifunctional megastigmanes in Boronia megastigma.

Furthermore, the first carotenoid-derived compounds with saturated ring could be identified and proven by synthesis in this substrate, namely the (E)-7-megastigmen-3,9-dione (40) and the (E)-3-hydroxy-7-megastigmen-9ones 41 and 42. In 1994, Weyerstahl (18, 41) confirmed these findings in his comprehensive paper on *Boronia megastigma* and identified additionally the (Z)-3-hydroxy-7-megastigmen-9-one (43). We could identify the bifunctional megastigmenes 40 – 42 also in the flower scent of *Masdevallia laucheana*, a fascinating Costa Rican orchid emitting its very diffusive rosy-floral and ionone-floral perfume only during the 30 or 40 minutes of twilight (1, 42). Furthermore, the same compounds 40 – 42 could be found by us on the peel surface of fully ripe lemon fruits (43) and the dione 40 in the flower scents of a *Mauriri* species (FH 4513, Melostomataceae) (20) and *Lithocarpus leucostachyus* (Fagaceae) (20).

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Carotenoid-derived Compounds in Michelia champaca

Also very rich in carotenoid-derived compounds is the flower scent of *Michelia champaca* L., a medium-size tree of the Magnoliaceae native to the temperate Himalayas which has found a wide distribution throughout the subtropical and tropical Eastern hemisphere. *Michelia champaca* (often just named Champaca) belongs together with the white-blooming *Michelia alba* (often named Michelia), several *Plumeria* species, *Jasminum sambac* (Malati, , Sampaquite) and *Hedychium coronarium* (White Ginger Lily) to the most famous fragrant flowers of South East Asia, all enjoying the status of ceremonial flowers.

In recent times, concretes and absolutes of Michelia champaca have become commercially available as expensive specialities for the creation of high-class fragrances. As our investigations (19) showed they are based on a common qualitative profile, they differ, however, considerably in their quantitative compositions. Thus, commercially available concretes showed ionone contents between 1 % and 7.8 % while a concrete prepared by ourselves 41 % (for the latter β -ionone 20 %, dihydro- β -ionone 10 %, α -ionone 6.8 %, dihydro- β -ionol 3.8 %, β -ionol 0.8 %). Some further quantitative differences suggest that these extract samples have been prepared from distinct subspecies or chemotypes, respectively. Quite interestingly, in two of the commercial concretes the oximes 47 - 50 derived from the main ionones (present in all samples) could be identified, while in others including the one prepared by us, they seem to be absent (cf. Figure 9). However, all extract samples evaluated during this investigation as well as the headspace samples of the flowers, which we also used for our own extract, contained the oximes 44 - 46 which are considered to be formed from the corresponding amino acids isoleucine, leucine The original and phenylalanine (cf. (1) and (42) and literature cited). hypothesis that these ionone oximes 47 - 50 are possibly formed by transoximation of these primary formed oximes with the respective ionones is questioned by the fact, that the reinvestigation of the same concrete samples after a storage time of two years did not show an increase or new formation of the oximes in question. Of interest in this context is also the identification of the 3-methyl-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)-isoxazole (51. **B**-ionone isoxazole) in the Champaca concrete, the well-known intermediate in the Büchi-Vederas transformation (44) of β -ionone (1) to β -damascone, which is accompanied by the corresponding epoxy-isoxazole 52.

For another nitrogen-containing constituent with elementary composition of $C_{13}H_{23}N$ the structure of 2,5,5-trimethyl-5,6,7,8-tetrahydroquinoline (55) could be proposed which was verified by synthesis (19). The diketone 53 (45) was converted to its dioxime 54 which cyclized spontaneously to the tetrahydroquinoline 55 (Figure 10). The latter compound is characterized by an interesting quinoline-like, leathery odor accompanied by ionone aspects.

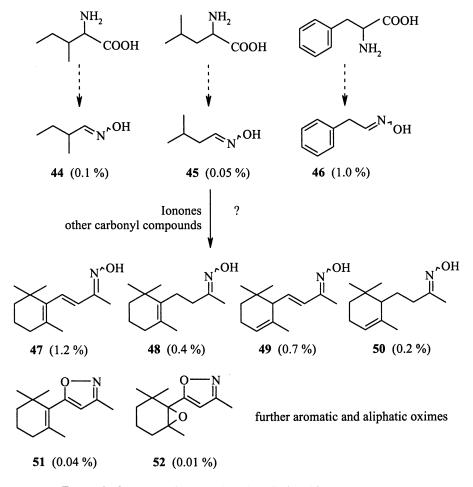


Figure 9. Oximes and isoxazoles identified in Champaca concrete.

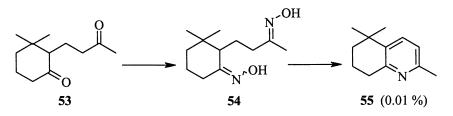


Figure 10. Synthesis of the tetrahydroquinoline 55.

As illustrated by Figure 11 the *Champaca* concrete contains also a series of bifunctional ionones (27, 56, 57) and derivatives thereof as the bicyclic dienone 58 known to occur in Burley tobacco (46) and yellow passionfruit (47) and the pyran derivative 59 also known to occur in tobacco (46). The dienone 58 is accessible by internal aldol condensation of 56 while 59 is easily formed by acid treatment of the hydroxyketone 57. Besides *Osmanthus fragrans* (13, 35) the four isomers of 7-oxo-dihydrospirane (35 a – d) have only been identified yet in *Michelia champaca*. The fact that all the *Champaca* constituents listed in Figure 11 also occur in *Osmanthus fragrans* (13) illustrates once again the enormous diversity of carotenoid-derived compounds in this unique flower scent.

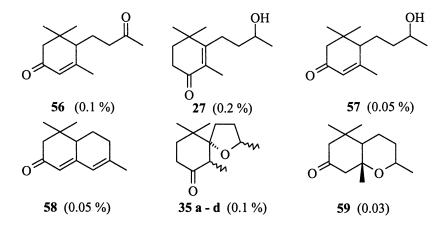


Figure 11. Derivatives of bifunctional ionones in Champaca concrete.

Carotenoid-derived Compounds in Rose Scents

Until the beginning of the 19th century, the classical European rose gardens have been totally dominated by the old famous fragrance roses *Rosa alba*, *R. gallica*, *R. centifolia* and *R. damascena*, which cover only white to red colors, caused mainly by flavonoids. Correspondingly, their scents contain, if at all, only very small amounts of carotenoid-derived compounds which are, however, still of eminent olfactory importance due to their low threshold values.

The discovery of one of these trace constituents, (E)- β -damascenone (5) (27), has even become a history case in natural scent research. It was found to be accompanied by even much smaller amounts of (E)- β -damascone (61) and the assumed natural precursor of 5, the 3-hydroxy- β -damascone (60) (48) (Figure 12). In certain cultivars of *Rosa damascena* and *R. centifolia* also small amount of β -ionone (1) (8, 20) and dihydro- β -ionone (2) (20) could be identified.

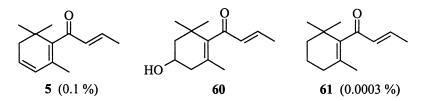


Figure 12. Carotenoid-derived compounds in Rosa damascena.

However, by far not all the over 150 rose species and thousands of cultivars derived from them display scents, which may be considered as being related to these famous perfumery roses. A very illustrating example give the old "China roses" which are mainly crosses between *Rosa gigantea* and *Rosa chinensis* both very rich in carotenoid-derived compounds, especially β -ionone (1), dihydro- β -ionone (2), β -ionol, dihydro- β -ionol and the theaspiranes (7, 20). They have been introduced at the beginning of the 19th century to the Western World to contribute tea-like, ionone-like, balsamic and green-herbaceous aspects to the scents of "Tea roses", "Noisette roses" and "Hybrid Tea roses" subsequently developed by crossing with the "Western roses".

A typical example is the famous yellow old garden rose "Maréchal Niel" (Noisette) which is – regarding appearance and scent – still close to one of its parents, to *Rosa gigantea* native of Yunnan (17). Its scent is olfactorily strongly dominated by carotenoid metabolites and 3,5-dimethoxy-toluene and has practically nothing in common with that of *Rosa centifolia*.

Since the beginning of the 20^{th} century, another intensively yellow colored rose species, rich in carotenoids, is additionally used in the breeding of Hybrid Tea roses, *Rosa foetida* native of Central Asia. Since this rose species does obviously not contain the cleaving enzymes, no carotenoid-derived compounds could be identified in its scent (17). This is in contrast to various other rose species, the old "China roses" and the Hybrid Tea roses containing the blood of "China roses", in which a catabolism of the carotenoids according to the "Eugster equation" (3) shown in Figure 13 may be observed.

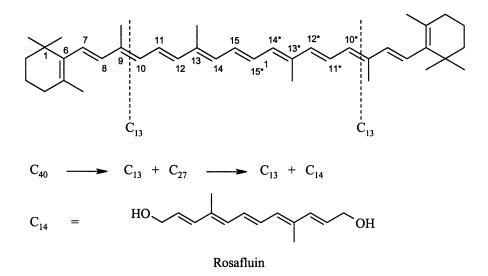


Figure 13. Catabolism of rose carotenoids (3)

As found by Eugster (3) the postulated Car $(9,10:9^*,10^*)$ dioxygenase is not specific to the end-groups but causes excentric cleavage leading to the corresponding C₁₃-metabolite and the C₂₇-alcohol. As further found in many cases by Eugster, the latter may be transferred to the C₁₄-diol Rosafluin and a second C₁₃-compound (Figure 13). The carotenoid-derived compounds identified by us in a series of old garden roses and Hybride Tea roses have been summarized in a paper entitled "The Chemistry of Rose Pigments" (3). A series of further papers describe these compounds as constituents of the scents of Hybride Tea roses (7, 10 - 11, 23, 49 - 50) and natural rose species (9, 51).

Carotenoid-derived Compounds in Orchid Scents

Investigating the flower scents of neotropic orchids we encountered several times the 7,11-epoxy-5(6)-megastigmen-9-one (**38**) as dominating constituent (1, 52), a compound formerly only known to occur as minor component of passionfruit flavor (40) and the flower scent of *Boronia megastigma* (17, 41). Thus, in the very strong ionone-floral scent of *Houlletia odoratissima* Lind ex Lindl. et Pax, a rare terrestrial orchid native to the Northern part of South America, we found **38** to be present to the astonishing high amount of 79 %, accompanied by two unidentified minor components of molecular weights 206 and 166, respectively. In the headspace trapping of the recently described *Gongora cruciformis* Whitten & D. E. Benn (53), we found **38** to around 38 % together with the unidentified compound of molecular weight 166 (1,8 %) from which the latter could be isolated and elucidated as the 7,7-dimethyl-4,5,6,7-tetrahydro-1(3H)-isobenzofuranone (**65**) (52).

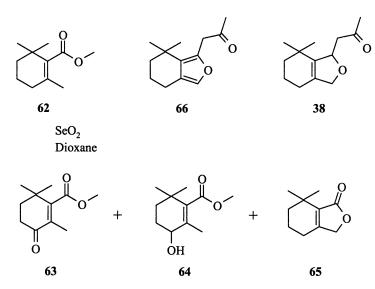


Figure 14. 7,11-Epoxy-5(6)-megastigmen-9-one and its degradation product 65.

This structure was verified by selenium dioxide oxidation of methyl β -cyclogeranate (62) (54) leading to the bicyclic lactone 65 together with the keto ester 63 and hydroxy ester 64 in a ratio of 1 : 7 : 2 (Figure 14). For the accompanying compound of molecular weight 206 the structure of the 7,11-

epoxy-5(7),6(11)-megastigmadien-9-one (**66**) could be proposed which has, however, not yet been confirmed by synthesis.

The orchid family with its extreme diversity in ecological groups and olfactory notes connected to them (1) may be regarded in a first approximation as representing the floral scent chemistry of the entire kingdom of flowering plants. Among the around 3500 species of Orchidaceae evaluated by the author in the course of the past 20 years around 10 % showed olfactory notes reminiscent of ionones and among the 600 representatives also analytically investigated, 70 species contained in their scents β -ionone (1) and 60 species dihydro- β -ionone (2). Taking into consideration, that among the total of 1250 species of scented flowers investigated by the author, 16 % contained β -ionone, it can be estimated that carotenoid-derived compounds may be found in the scents of at least 30,000 species of flowering plants.

The occurrence of some other interesting derivatives of β -ionone in orchid scents as the photoisomers of β -ionone **36** and **37** and the ring hydrogenated bifunctional derivatives **42** and **43** have already been discussed. It does not surprise that orchid scents also contain carotenoid-derived compounds with 10 and 9 carbon atoms (Figures 15 and 16). Thus, the scent of the neotropic species *Cattleya schilleriana* (20) contains besides ionones β -cyclocitral (74) and that of *Encyclia radiata* (20) safranal (75) while that of *Encyclia baculus* (1) is very much dominated by the oxoisophorone **67**, accompanied by its derivatives **68** – **70**. The compounds **67**, **69** and **74** have been described as occuring in the flower scent of *Jacquinia species* (55) and **67** and **69** – **73** in that of common cherry

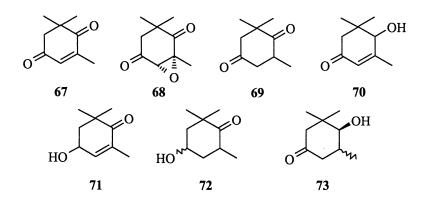


Figure 15. Isophorone derivatives found in various flower scents.

laurel (*Prunus laurocerasus*) (56). Furthermore, most of the compounds 67 - 75 have been found in saffron (57 and lit. cited).

Carotenoid-derived Compounds in Various Flower Scents

Considering the enormous distribution of carotenoid-derived compounds in flower scents it was not possible to give an overview on all natural occurrences in this paper. The intention was more to illustrate with the most exemplary floral scents the broad spectrum of natural compounds which may be generated by carotenoid catabolism. In this sense the following concluding examples should also be understood. Thus, in the headspace trapping of the flower scent of *Anthurium salvadorense* (Araceae) (20) the β -cyclocitral (74, 1 %) and safranal (75, 0.1 %) are accompanied by β -cyclogeraniol (76, 0.6 %) and the new natural products β -safranol (77, 0.2 %) and β -safranyl acetate (78, 0.1 %) (Figure 16).

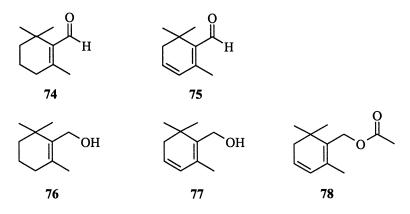


Figure 16. Derivatives of β -cyclocitral (74) and safranal (75) in flower scents.

Safranal (75) and β -safranyl acetate (78) have been found by us also in the floral scent of a *Palisota* sp. (GHS006) (Commelinaceae) native to the region of La Makandée in Gabon (20).

In the headspace concentrate of the ionone-floral scent of *Reseda odorata* remarkable amounts of 8,9-dehydro-4,5-dihydrotheaspirone (**79 a/b**, 4 - 11 %) and 8,9-dehydrotheaspirone (**80**, 4 - 6 %) could be identified (*56*). We found the two isomers **79 a** and **79 b** in a ratio of around 10 : 1 together with the (*E*)-7-megastigmen-3,9-dione (**40**) in the trapped scent of a *Mauriri* species (FH4516, Melostomataceae) native to French Guiana (*20*).

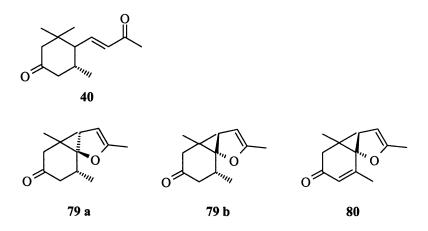


Figure 17. Theaspiranones identified in flower scents.

Compounds 79 a/b and 80 were identified some time ago as aroma constituents of tobacco (58 - 59) and more recently 80 was identified as an aglycon in Riesling wine (60).

Substituting the great number of not cited papers describing the ionones including derivatives as constituents, the following three are adequate to conclude this overview on carotenoid-derived compounds in flower scents. Lawsonia inermis (Lythraceae), the Henna already lauded by King Solomon, contains up to 48 % β -ionone (22), Telosma cordata (Asclepiadaceae) is rich in the theaspiranes, dihydro- β -ionone and β -ionone (21) and is well known on the Hawaii Islands under the name pakalana as ceremonial flower, and finally Hedychium coronarium (Zingiberaceae), the utmost sensual White Ginger Lily, contains only small but olfactorily significant amounts of β -ionone (61).

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Chapter 13

Bound Volatiles in Brown Boronia Flowers (Boronia megastigma)

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Precursors to twelve norisoprenoids were quantified throughout 48 h of incubation of brown boronia flowers at 12 and 30°C after excision from the plant, conditions under which the tissue concentrations of free norisoprenoids have been shown to increase. Bound norisoprenoids were released from two precursor extracts of differing polarity by hydrolysis with either β -glucosidase or endogenous enzyme fractions from brown boronia (Boronia megastigma Nees) flowers, the activities of which were also monitored throughout incubation. 'Norisoprenoid-producing' activity of boronia enzyme fractions increased during early stages of incubation, co-incident with a decline in the concentration of precursors. most norisoprenoids hydrolyzed Precursors to were exclusively, or to a greater extent, by boronia enzymes compared with β -glucosidase. This includes β -ionone, which was repeatably found after incubation with boronia enzymes. Throughout incubation of flowers, the nature of the bound precursors changed, becoming more polar and more easily hydrolyzed by β -glucosidase, suggesting a shift to a less complex moiety. Little overall change in glycosidase activity within boronia enzyme fractions occurred. It is likely that hydrolysis of complex, water soluble precursors explains at least some of the increases in free norisoprenoids observed in boronia flowers after harvest.

Brown boronia flowers (*Boronia megastigma* Nees, family Rutaceae) are grown for their highly valued fragrance, comprising amongst other compounds, at least 24 norisoprenoids including β -ionone (up to 0.1% fresh weight) (*1-4*). After removal from the plant, the concentration of floral volatiles increases significantly, a process which is regulated by environmental, physiological and genetic factors (*1-3*). Maximum postharvest increases in β -ionone occurred in flowers that had opened within the last 2-3 days; an increase in incubation temperature from 12 to 23°C reduced the time to maximum increase (14 compared with 24 h) (*1*). Postharvest production of β -ionone was inhibited by 2 mM NaN₃, 600 μ M 2,4-DNP, 3 mM KCN and in atmospheres lacking O₂ (*3*). Genetically different plants generated between 45-181% more β -ionone during 24-48 h incubation at 12°C; an example of the variation is illustrated in Table I.

Plant type:	Α	В	С	D
Initial conc. (% fr. wt.)	0.114	0.067	0.053	0.055
Final conc. (% fr. wt.)	0.165	0.128	0.108	0.155
Time to max. (h)	24	36	48	48
% increase	45	91	103	181

Table I. Concentration of β -ionone before and after postharvest incubation at 12°C of four genetically different plant types (clones A-D).

Research by our team suggests that the process generating β -ionone, amongst other volatiles, requires energy and is enzymatic. In immature and senescent flowers either the precursor is absent or the enzyme is inactive, as no increase occurs. Subsequent to the maximum concentration occurring, the concentration of volatiles declines. Analysis of norisoprenoids released after incubation of precursor solutions, made by methanolic extraction and C18 separation, with β -glucosidase and fractionated boronia enzymes has been used to examine the identity of precursors to norisoprenoids and to characterize the 'norisoprenoid-producing' enzyme(s). Boronia flowers of optimum maturity for postharvest production of free norisoprenoids were incubated at 12 and 30°C for up to 48 h. The concentration of norisoprenoid precursors and activity of enzymes were monitored throughout.

Experimental Procedures

Flower material. Clonal plants of *B. megastigma*, developed by the University of Tasmania and grown on commercial plantations in Tasmania were used. Flowers were harvested at 80% open flowers (5) using hand-held combs and were either a) immediately made in to acetone powders; b) were frozen at -18° C until precursors were extracted with methanol; or c) were incubated at 12 and 30°C for up to 48 h followed by a) and b).

Enzyme Activity. Acetone powders were prepared by blending fresh flowers with chilled acetone (-18°C) in a stainless steel blender. The homogenate was filtered under vacuum using Whatman #4 filter paper; the solids were washed several times with chilled acetone until the filtrate was clear, then spread over filter paper and left to air dry until all traces of acetone disappeared. The powders were stored at 4°C until required (approx. 11%) protein g/g). Acetone powders were resolubilized in 0.05 M acetate buffer pH 5, 1:10 g/ml, with 0.1% v/v Tween 80 and an equal weight of PVPP, overnight at 4°C. After filtration under vacuum the filtrate was fractionated into three parts by addition of ammonium sulfate to 0-45%, 46-65% and 66-80% saturation. Protein pellets collected by centrifugation for 30 min at 18,000 rpm (7°C) were dissolved in buffer and the protein contents assayed using the Bio-Rad Protein Assay (BioRad Laboratories, Richmond, CA), used as per the manufacturer's instructions, with BSA as a standard.

Each enzyme fraction was analyzed for galactosidase activity by incubation with commercial para-nitrophenyl- β -D-galactopyranoside (25 mM) at 30°C for 30 minutes followed by addition of 0.2 M Na₂CO₃; the adsorption was measured at 420 nm. The synthetic glycoside para-nitrophenyl- β -D-galactopyranoside was previously observed to be the glycoside most actively hydrolyzed by boronia enzymes from a selection of α -galactosides, β -glucosides and α -mannosides.

Each enzyme fraction was analyzed for 'volatile producing activity' by incubation with two precursor fractions from a standard methanolic extract made from boronia flowers. The standard precursor extract was prepared by blending frozen boronia flowers in a stainless steel blender, followed by removal of volatiles and pigments with petroleum ether, and subsequent extraction of precursors in methanol. The methanol extract was concentrated under reduced pressure at 40°C, re-extracted with petroleum ether and diethyl ether, re-dissolved in methanol and filtered. The so-prepared precursor extract was passed through several C18 Sep-Pak filters in sequence that were subsequently washed with methanol and water. Two precursor fractions of differing polarity were prepared by elution in 30% (A) and 60% (B) ethanol; each eluted fraction was dried down at 40°C under reduced pressure and resolubilized in buffer prior to enzymatic hydrolysis. Aliquots of each enzyme fraction, and β -glucosidase (almond emulsin, 4-5.6 units/mg, Sigma), were separately incubated with both precursor fractions at 37°C for 24 h. The reactions were stopped and the free volatiles extracted by addition of CH₂Cl₂ containing an octadecane internal standard, overnight at 4°C. The CH₂Cl₂ layer was removed and analyzed directly by GC/MS. The methodology for both enzyme fractionation and preparation of bound volatile fractions were developed after extensive experimentation (6).

Concentration of Precursors. Methanolic extractions of precursors in flowers incubated after harvest were assayed as above via incubation with standard enzymes prepared by solubilization of acetone powders without fractionation with ammonium sulfate. Both A and B (precursor fractions) were incubated alone, with β -glucosidase and with the standard boronia enzyme(s) for 24 h at 37°C; the reactions were stopped and free volatiles extracted and analyzed as above.

GC/MS Analysis. A HP 5890 GC coupled to a HP 5970B Mass Selective Detector (MSD) via an open-split interface was used to analyze 1 µL injections of the CH_2Cl_2 layer using a HP-1 column (25 m x 0.32 mm internal diameter, $0.52 \ \mu m$ film thickness). The carrier gas was helium at 4 mL/min, the source temperature was 200°C and the electron energy 70 eV. The injection temperature was 240°C, the oven temperature was 60-120°C at 20°C/min, 120-210°C at 6°C/min, 210-290°C at 15°C/min. Free volatiles were identified by a combination of MS and Kovats indices (4,7). The total ion current area (TIC) for each compound was determined by integrating a peak at the appropriate retention time in a mass chromatogram of a suitable diagnostic ion, this area was scaled up by the factor representing the proportion of the diagnostic ion of the TIC of a 'clean' mass spectrum for each compound. In this way, partially resolved peaks could be readily estimated. The total ion current for each volatile was related to the total ion current for the octadecane internal standard, and calculations made on the basis of the amount of flower material used for each sample. Individual boronia enzyme and precursor fractions were also incubated (separately) and the volatiles released from these fractions, if any, were

quantified and subtracted from combined incubations. Each precursor fraction was likewise incubated with β -glucosidase.

Results and Discussion

Of the 35 compounds observed after hydrolysis of precursor fractions, there were 12 norisoprenoids, 11 of which were identified by GC/MS and Kovats indices (Table II), 9 of which were previously documented (4). Changes in the 'norisoprenoid-producing' activity of three boronia enzyme fractions were assessed by incubation with two standard precursors, prepared by passing a methanolic extract through a C18 column and eluting with 30 and 60% ethanol precursors (fractions A and B, respectively). Changes in the activity of the three enzyme fractions, 1, 2 and 3 (0-45%, 46-65% and 66-80% ammonium sulfate saturation) at releasing free β -ionone from A and B are illustrated as a general example (Figure 1). During the first 12 h of incubation, there was a rapid increase in activity in enzyme fractions 1 and 2 against precursor fraction B, which then rapidly declined (Figure 1, top). After 40 h, there was a second increase in activity in all enzymes, especially at 30°C. Enzyme 1 was also active against precursor fraction II, increasing after 12 h and subsequently declining steadily (Figure 1, bottom). The greatest activity against precursor B occurred in enzyme 1 after 24 h incubation (Figure 1, bottom).

In general, higher activity, measured as total ion current for each volatile per milligram (TIC/mg of flower material) released from a standard precursor, occurred in flowers incubated at 30°C compared with 12°C (data not shown). A comparison of the activity of each enzyme fraction at releasing each of the 12 norisoprenoids is presented in Table II. It shows the predominance of 'norisoprenoid-producing' activity for most norisoprenoids in enzyme 2 (46-65% ammonium sulfate). In addition, for norisoprenoids marked with an 'a' in Table II, release of the free volatile occurred only after treatment with boronia enzyme fractions, not upon incubation with β -glucosidase. The glycosidase activity of each boronia enzyme fraction was also assessed by incubation with synthetic glycosides (Figure 2). Activity against pNP-β-D-galactopyranoside was previously demonstrated to be highest relative to hydrolytic activity against other glycosides (data not shown). Comparison of Figures 1 and 2, particularly the lack of change in glycosidase activity in enzyme 3 and the decline in enzyme 2, shows that the changes do not follow the same patterns in the, potentially, two different enzyme 'groups'. Thus enzymes producing free norisoprenoids from precursor solutions may not by glycosidases, or may hydrolyze more complex moieties than galactosides. The total concentration of protein in boronia flowers decreased throughout post-harvest incubation, the decrease being more pronounced at 30°C (data not shown).

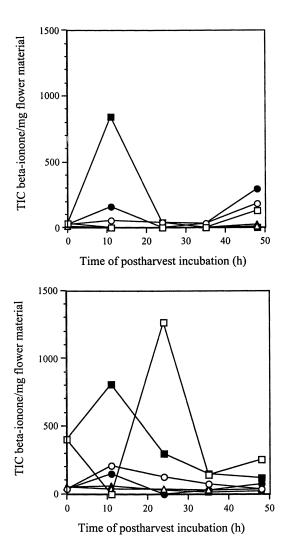


Figure 1. Change in activity of 3 boronia enzyme fractions during postharvest incubation – release of \$\beta\$-ionone from standard precursors A (top) and B (bottom). Enzyme fraction 1 (squares), enzyme fraction 2 (circles), enzyme fraction 3 (triangles); flowers, from which enzyme fractions made, previously incubated at 12°C (unfilled symbols) and 30°C (filled symbols).

No. ^b		Compound	TIC/g fle Fraction	ower materia	ıl°
			1	2	3
8	а	5,6-epoxy-β-ionone	10	322	91
9	а	β-ionone	0	291	24
10	а	dihydroactinidiolide	0	244	157
11	а	unknown (m/z 151,194)	0	141	344
12		7,11-epoxy megastigma-5- en-9-one	41	425	541
14	а	3-oxo-5,6-dihydro-β-ionone	20	0	136
17		(<i>E</i>)-3-hydroxy-5,6-dihydro- β-ionone	1002	7845	2588
19		4-oxo-β-ionone	30	958	560
20		4-hydroxy-β-ionone	189	2467	2800
21		4-oxo-β-ionol	0	459	486
22		5,6-epoxy-3-hydroxy-β- ionone	20	256	126
24		3-hydroxy-β-ionone	259	3000	2447

Table II. Twelve norisoprenoids quantified using total ion current (TIC/g flower material) after hydrolysis from standard precursor fraction A (30% ethanol) by enzyme fractions 1 (0-45%), 2 (46-65%) and 3 (66-80% ammonium sulfate) from flowers incubated for 48 h at 30°C.

^aCompound was released exclusively after treatment with boronia enzymes. ^bNumbering refers to elution from GC column, a comprehensive list will be published elsewhere. ^cTIC/g units from chromatogram integration were simplified by removal of three digits at the end of each number, and rounded up.

N.B. Due to the means of quantifying compounds, comparison of TIC/g may only be made within results for each compound, not between different compounds.

The predominant form in which norisoprenoid precursors were bound changed throughout incubation (Table III). At harvest, most precursors were hydrolyzed most effectively by boronia enzymes, and were present in precursor fraction B. After the first 24 h of incubation, many of the precursors occurred in fraction A, and glucosides (i.e. precursors hydrolyzed by β -glucosidase) of 12, 19 and 21 occurred for the first time. In the second 24 h of incubation, precursors of 8, 9, 10, 11, 14, 19 and 21 were depleted, and generally glucosides predominated over other bound forms, the latter distinguished by hydrolysis with boronia enzymes compared with β -glucosidase (Table III). One possible explanation for this change would be a systematic hydrolytic process by the more complex boronia enzymes, initially cleaving moieties which change the polarity of the precursor, and later leaving a glycoside more easily hydrolyzed by β -glucosidase.

Table III. Changes in predominant form in which norisoprenoid precursors occur in boronia flowers throughout post harvest incubation at 12°C.

		IIar	narvest incubation at 12°C.	
	No. a	Zero time	24 h (12°C)	48 h (12°C)
	×	bound ^b (B)	bound (A)	Nil
	6	bound (B)	bound (A)	Nil
	10	gluc ^c (A)	bound (A)	Nil
		bound (B)		
	11	gluc (A)	bound (A)	Nil
		bound (B)		
	12	bound (B)	bound > gluc (A)	gluc (B)
			gluc (B)	1
	14	bound (B)	bound (A)	Nil
	17	bound < gluc (A)	bound > gluc (A)	gluc (A)
		bound $>$ gluc (B)	gluc (B)	gluc > bound (B)
	19	bound (B)	bound $>$ gluc (A)	Nil
	20	gluc (A)	bound $>$ gluc (A)	gluc (A)
		bound >gluc (B)	gluc (B)	bound (B)
	21	bound (B)	bound $>$ gluc (A)	Nil
	22	gluc (A)	bound $>$ gluc (A)	gluc (A)
		bound (B)		
	24	gluc (A)	bound > gluc (A)	gluc (A)
		bound $>$ gluc (B)	gluc (B)	bound (B)
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The changes observed in enzyme activity in each enzyme fraction against each of the two precursors (Figure 1) also help to explain the change, with initially high activity against precursor A, and later activity against precursor B. Overall, there was probably little change in total glucosidase activity, despite changes in activity in specific fractions (Figure 2).

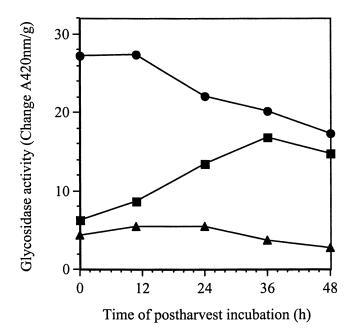


Figure 2. Glycosidase activity (change in A420nm/g fresh weight) in three enzyme fractions from flowers incubated at 12°C after harvest; enzyme fraction 1 (squares), enzyme fraction 2 (circles), enzyme fraction 3 (triangles).

'Quantitative' comparison of enzyme activity and concentration of precursors in flowers throughout post harvest incubation has been attempted (Table IV). The increase in enzyme activity between 0 and 24 h for most norisoprenoid precursors is apparent, with a subsequent decline in some cases. Correspondingly, a decline in the concentration of norisoprenoid precursors occurs between 0-24 h, and most decline totally in the subsequent 24 h period of incubation (Table IV).

Table IV. Comparison of enzyme activity and concentration of precursors in boronia flowers throughout postharvest incubation.

	-	Enzyme acuvuly	2	CURC. US	Jone. of precursors (110/mg	
	(TIC/mg	C/mg released by 3 enzyme	3 enzyme	released	released by 3 enzyme fractions	fractions
	fractions f	ractions from precursors A and B)	rs A and B)	from p	from precursors A and B)	ind B)
No.ª	0	24 h	48 h	0	24 h	48 h
∞	123	969	409	50	32	0
6	541	1453	645	84	63	0
10	195	644	369	73	48	0
11	20	39	162	158	114	0
12	187	389	270	31	33	0
14	277	402	384	62	31	0
17	3342	3372	7754	1495	961	18
19	223	1043	1035	108	85	0
20	164	422	945	269	152	0
21	219	579	403	45	25	0
22	27	107	22	60	37	0
24	428	1475	1841	274	177	0

Summary

Twelve norisoprenoids were observed after hydrolysis of precursor fractions with enzymes, both prepared from boronia flowers throughout postharvest incubation. The activity of enzymes that hydrolyzed norisoprenoid precursors, releasing the free volatile, increased during the first 12 h of incubation, subsequently decreasing and again increasing in the final 12 of 48 h incubation. The concentration of bound norisoprenoids declined during incubation, with a gradual shift from less polar to more polar forms, and from complex bound forms to more simple glucosides. It is therefore likely that hydrolysis of bound forms, or norisoprenoid precursors, may explain the increase in norisoprenoids and other volatiles during postharvest incubation. The structure of the precursors and exact nature of the hydrolytic activity of the active enzymes remains the subject of future work.

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C₁₃-Norisoprenoids in the Aroma of Colombian Tropical Fruits

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This chapter focuses on the identification of C₁₃-norisoprenoid volatile compounds in free and glycosidically bound forms in lulo (Solanum quitoense), lulo del Chocó (Solanum topiro) and Thirteen mammee apple (Mammea americana). C13norisoprenoids were identified in lulo plant (pulp and peelings of the fruit, flowers and leaves) in free and bound forms. Furthermore, by chromatographic and spectroscopic means we could identify in lulo leaves the new compound (6R,9R)-13hydroxy-3-oxo- α -ionol 9-O- β -D-glucopyranoside, the known (3S,5R,8R)-3,5-dihydroxy-6,7-megastigmadien-9-one 5-O-B-9-O-β-D-gluco-D-glucopyranoside, (6S.9R)vomifoliol pyranoside, (3S)-3-hydroxy-5,6-epoxy-β-ionol 9-O-β-D-glucopyranoside and (6R,9R)-3-oxo- α -ionol 9-O-β-D-glucopyranoside. The role of the second mentioned glucoside as a precursor of β-damascenone and 3-hydroxy-β-damascone was also established. With the aid of capillary GC and capillary GC-MS (EI, NCI) of TFA glycoside derivatives, several C_{13} norisoprenoid glucoconjugates including the novel 3,6-epoxy-7-megastigmene-5,9-diol β-D-glucopyranoside could be identified in lulo del Chocó and mammee apple.

 C_{13} -Norisoprenoids are important carotenoid metabolites that contribute to the overall flavor of many fruits, such as ,e.g. grapes, (*Vitis vinifera*) (1,2), rasp-

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berry fruit (*Rubus idaeus*) (2), starfruit (*Averrhoa carambola*) (2,3), passion fruit (*Passiflora edulis*) (2,4), quince fruit (*Cydonia oblonga*) (2,5), and apples (*Malus* sp.) (6). During our continuous studies on the aroma of Colombian tropical fruits such as mountain papaya (*Carica pubescens*) (7), curuba (*Passiflora mollissima*) (8), tamarillo (*Cyphomandra betacea*) (9), granadilla (*Passiflora vitifolia*) (10), Andes berry (*Rubus glaucus*) (11), guava (*Psidium guajava*) (12), piñuela (*Sicana odorifera*) (13), we have noticed that although they contain a high amount of carotenoids, the presence of C₁₃-norisoprenoids as free aroma compounds is rather scarce. In contrast, when we studied the flavor of lulo (*Solanun quitoense*), lulo del Chocó (*Solanum topiro*) and mammee apple (*Mammea americana*) we found a large number of structurally related C₁₃-compounds. This paper summarizes our recent studies on the free and glycosidically bound C₁₃-compounds isolated from these fruits.

EXPERIMENTAL PROCEDURE

Plant Materials and Chemicals. Lulo (Solanaceae, Solanum quitoense L.) harvested through 1991-1998, lulo del Chocó (Solanaceae, Solanum topiro) harvested in 1998, and mammee apple (Gutiferae, Mammea americana) harvested in 1996, were obtained from commercially grown cultivars located in Colombia. Voucher specimens are deposited at Instituto de Ciencias Naturales de la Universidad Nacional de Colombia.

All solvents used were of high purity at purchase (Merck) and were redistilled before use.

Isolation of Free Volatiles. Fully ripe fruits (*ca.* 1 kg) free of seeds and peelings (or 100 g in the case of peelings, flowers and leaves) were cut, homogenized and centrifuged at 10.000 rpm for 30 min. The filtered supernatant was subjected to continuous liquid-liquid extraction with pentanedichloromethane (1 :1, v/v) for 48 hours (14). The organic phase was dried over anhydrous sodium sulfate, concentrated using a Vigreux column (36°C) to 0.2 mL and subjected to capillary GC and capillary GC-MS.

Isolation of Glycosidically Bound Compounds. The pulp of each fruit (1.0 kg) separated from the peelings and the seeds (or 100 g in the case of peelings, flowers and leaves) was homogenized in 0.2 M phosphate buffer (pH 7.0) and centrifuged at 10.000 rpm for 30 min. The supernatant was passed through an Amberlite XAD-2 resin according to a procedure published by Günata *et al.* (15). After rinsing the column with water, elution was performed

with methanol. The eluate was concentrated to dryness under reduced pressure. Part of the glycosidic extract (100 mg) was redissolved in 50 mL of 0.2 M citrate-phosphate buffer (pH 5.0) and incubated with 300 μ L of Rohapect D5L (Röhm) at 37°C overnight in the presence of phenyl β -D-glucopyranoside as internal standard (1 mg/kg). The liberated bound compounds were extracted with diethyl ether and the organic phase was dried over anhydrous sodium sulfate, concentrated (Vigreux column, 36°C) to 0.2 mL, and subjected to capillary GC and capillary GC-MS analysis.

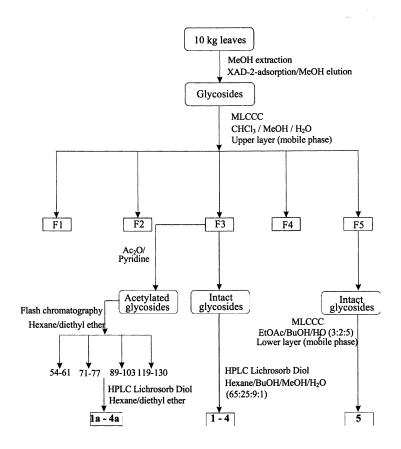


Figure 1. Protocol for the isolation of C_{13} -glycosides from lulo (Solanum quitoense) leaves.

In Carotenoid-Derived Aroma Compounds; Winterhalter, P., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2001. Isolation and Identification of Glycoconjugates in Lulo (S. quitoense) Leaves. A protocol for the isolation of glycoconjugates of lulo leaves is depicted in Figure 1.

Fresh lulo leaves (10 kg) were blended and subjected to glycoside extraction as mentioned above to afford 15 g of crude glycosides. Portions of ~ 1.5 g of this extract were placed in an Ito multilayer countercurrent chromatograph (MLCCC, P.C. Inc., Potomac, MD) with 75 m x 2.6 mm i.d. PTFE tubing (total volume = 400 mL) for separation of glycosides. The MLCCC apparatus was operated at a rotation speed of 800 rpm, using $CHCl_3$ /MeOH/H₂O (7:13:8) as solvent system with the less dense layer as mobile phase at a flow rate of 1 mL/min. Fifty fractions (each 10 mL) were collected. Half of the combined fraction F3 (13-16) was concentrated and finally purified by preparative HPLC using a Lichrosorb Diol column (5 µm, 250 x 4.6 mm i.d., Merck) with hexane/BuOH/MeOH/H₂O (65:25:9:1) (flow rate 1 mL/min) as solvent system to yield pure glycosides 1-4. The other half of F3 was peracetylated with acetic anhydride and pyridine and the resultant peracetylated glycosides were further purified by SiO, flash chromatography (hexane/diethyl ether, 1:5) and preparative HPLC using a Lichrosorb Diol column with hexane/diethyl ether (1:1) as mobile phase to give pure peracetylated compounds 1a-4a. Combined fraction F5 (40-50)was refractionated by MLCCC with the lower phase of EtOAc/BuOH/H₂0 solvent system as mobile phase, to obtain pure glycoside 5.

The identification of glycosides 1-4, of their corresponding acetates 1a-4a and of glycoside 5, respectively, were performed by spectroscopic analysis. UV spectra were obtained with a Merck Hitachi diode array detector L-4500, FAB-MS spectrometry was carried out with a JEOL JMS-AX505HA spectrometer using glycerol matrix, CI-MS with a Shimadzu 9020 DF mass spectrometer and NMR spectra were taken on Fourier transform JEOL JNM-EX500 and Bruker AC-500 spectrometers with Me₄Si as internal standard.

Isolation and Identification of Glycoconjugates as TFA Derivatives in Lulo del Chocó (S. topiro) and Mammee Apple (M. americana) Fruits. Part of the glycosidic extract of the fruit pulp of each fruit (100 mg) was derivatized with 100 μ l of TFA reagent [N-methyl-bis-(trifluoroacetamide)] in 100 μ L of pyridine, according to Voirin *et al.* (16) using phenyl β -D-glucopyranoside as internal standard. The TFA derivatives thus obtained were subjected to capillary GC and capillary GC-MS analyses under EI and NCI conditions.

Capillary GC Analyses. Capillary GC analyses of free and bound volatiles were performed on a Hewlett Packard 5890 Series II gas

chromatograph equipped with FID detector and split/splitless injector, both operating at 250°C. A J&W fused silica DB-Wax capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness) was used with the following temperature program: 3 min isothermal at 50°C, then raised to 220°C at 4°C/min, and finally held at 220°C for 10 min. The flow for carrier gas was 1.1 mL/min He. Volumes of 1µL were injected with a split ratio of 1:15. Temperature programmed retention indices were determined using n-alkanes (C_{9} - C_{32}) as standards. For the calculation of retention indices on DB-5 we used a fused silica column (J&W Scientific Inc.) (25 m x 0.25 mm i.d., 1 µm film thickness) with the following temperature program: 4 min at 50°C then raised to 300°C at 4°C/min. Quantitative data for free and bound volatiles were obtained by the internal standard method using n-decanol and phenol (released by enzymatic hydrolysis of phenyl β -D-glucopyranoside), respectively, as reference substances without considering calibration factors, i.e. F = 1.00 for all compounds.

For trifluoroacetylated (TFA) glycosides analyses the same DB-5 fused silica column as described above was used. The column temperature was programmed at 3°C/min from 125 to 280°C and kept at this temperature for 10 min. The injector and detector temperatures were 280 and 300°C, respectively. Carrier gas flow and split injection were the same as those mentioned above. Retention indices were calculated using n-alkanes as standards and quantitative data were obtained using TFA phenyl β -D-glucopyranoside as internal standard.

Capillary GC-MS Analyses. GC-MS analyses of free and bound volatiles were carried out on a Hewlett Packard 5970 mass selective detector directly coupled to an HP 5890 gas chromatograph. The same type of column and temperature conditions as mentioned above for capillary GC analysis was used; electron energy 70 eV; mass range 30-300 for free volatiles and aglycons. Qualitative analysis (mass spectral studies) was verified by comparing retention indices and mass spectra of identified compounds with those of authentic reference substances or with published data (EPA/NIH library).

GC-MS analyses of TFA derivatives were carried out on a Shimadzu QP 5050 using EI and NCI. The same type of column and temperature conditions as mentioned above for capillary GC analysis was used. The operating conditions in the EI-MS mode were as follows: source temperature 200°C, mass spectra were scanned at 70 eV in the m/z range 69-900. NCI-MS of TFA glycosides was performed under the following conditions: temperature source 200°C; pressure of methane as reactant gas: 2.4 x 10^{-2} Pa, measured at the ion gauge; mass range m/z 100-900.

RESULTS AND DISCUSSION

C₁₃-Norisoprenoids in Lulo (S. quitoense) Plant

Table I shows the C_{13} -norisoprenoid compounds identified by capillary GC and capillary GC-MS analyses in the free and bound volatile extracts obtained from the pulp and peelings of lulo fruit and from its flowers and leaves. Table I also shows the retention indices found on DB-5 and DB-Wax columns, as well as the concentration of each volatile calculated on the basis of the standard added. Identifications were done using both chromatographic and spectral criteria described in the experimental procedure.

As can be seen from Table I, a total of thirteen C_{13} -norisoprenoids were identified in lulo plant sharing one characteristic feature. They are all oxygenated at carbon atom-3. 3-Oxo- α -ionol, 3-hydroxy-5,6-epoxy- β -ionol, (6S,5R)-vomifoliol, and (6R,9R)-13-hydroxy-3-oxo- α -ionol were identified as major components. Table I also reveals that a large proportion of C_{13} norisoprenoid compounds is present in glycosidically bound form, the highest abundance being found in the leaves and flowers of the plant.

On the basis of the above mentioned results, we proceeded to isolate and identify the intact C₁₃-norisoprenoid glycoconjugates in lulo leaves. A glycosidic extract from the leaves was obtained by Amberlite XAD-2 adsorption and methanol elution. The glycosidic isolate was subjected to MLCCC and HPLC as outlined in Figure 1 in order to obtain intact glucosides 1-5 and peracetylated glucosides 1a-4a. The spectroscopic studies of the aforementioned glucosides (intact and peracetylated) done by UV, MS, as well as by ¹H and ¹³C-NMR one - and two dimensional spectroscopy led to the identification of the novel (6R,9R)-13-hydroxy-3-oxo- α -ionol 9-O- β -D-glucopyranoside 1, the rare (3S,5R,8R)-3,5-dihydroxy-6,7-megastigmadien-9-one 5-O- β -D-glucopyranoside 2, as well as three common glucosides, i.e. (6S,9R) vomifoliol 9-O- β -D-glucopyranoside 4, and (6R,9R)-3-oxo- α -ionol 9-O- β -D-glucopyranoside 5 (cf. Figure 2). Details concerning the identification procedure of glucoconjugates 1-5 have been published elsewhere (17, 18).

Additionally, we submitted separately glucoconjugates 2 and 2a (in the latter case after deacetylation) to treatment with emulsin to yield as

Table I. Norisoprenoids Identified in Aerial Parts of Lulo (Solanum quitoense) Plant.	in Aerial	Parts of Lulo	o (Solanu	im quitoer	ise) Plan	lt.			
Compound		KI _{exp}	Ъ.	Pulp	Pe	Peelings	Flowers	Leaves	
1	DB-5	DB-Wax	Free	Bound ^a	Free	Bound ^a	Bound ^a	Bound ^a	
β-ionone	1497		‡						
dihydroactinidiolide	1552	2340	‡		‡				
3-oxo-α-damascone		2433	ı		‡		ı	ı	
3-oxo-α-ionol	1665	2639	‡		ı	‡	+ + +	+++++++++++++++++++++++++++++++++++++++	
3-hydroxy-5,6-epoxy-ß-ionol	1685	2767	+	+	ı	‡.	‡	+++++++++++++++++++++++++++++++++++++++	
3-hydroxy-7,8-didehydro-β-ionone	1689	2640	, I L	ı	+ + +	•	ı	+	
3-hydroxy-5,6-epoxy-β-ionone	1695	2668	+ + +		‡	·	ı	‡	
3-hydroxy-β-ionone	1709		‡						
(Z) -3-oxo-retro- α -ionol	1722	2741	ı	ı	1	‡	‡ + +	+ + +	
(E)-3-oxo-retro- α -ionol	1788	2875	•	+	ı	‡	ı	+ + +	
(6S,9R)-vomifoliol	1806	2932	ı	+	ı	+	۹ +	+ + +	
dehydrovomifoliol	1810		+	ı	•	1	ı	ı	
$(6R,9R)$ -13-hydroxy-3-oxo- α -ionol	1941			۹++		۹+	۹+	++++	
+ = < 50 μg/ kg; $++ = 50-500$ μg/kg; $+++ = 500-1500$ μg/kg; $++++ = > 1500$ μg/kg; $- =$ non detected, ^a Glycosidically bound C ₁₃ - norisoprenoid. ^b Detected as TFA derivative of the corresponding glycoside.	+++ = 50 ive of the 6	D-1500 μg/kg; corresponding g	++++ = > glycoside.	• 1500 µg/	kg; - = n	on detected,	^a Glycosidica	lly bound C ₁₃ -	

In Carotenoid-Derived Aroma Compounds; Winterhalter, P., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

enzymatically released product, 3-hydroxy-7,8-didehydro-β-ionone **6** (Figure 3). The structure of the aglycon was confirmed by capillary GC, capillary GC-MS and ¹H-NMR analyses. Further model reactions depicted in Figure 3 (NaBH₄ reduction of compound **6** and its subsequent heat-induced rearrangement under acidic conditions) revealed compound **2** as a new precursor of β-damascenone **8** and 3-hydroxy-β-damascone **9**. Ketone 1 is of considerable importance for the flavor and fragrance industry. It is also to be stressed that 3,5-dihydroxy-6,7-megastigmadien-9-one 5-O-β-D-glucopyranoside **2** is the genuine precursor of 3-hydroxy-7,8-didehydro-β-ionone **6**, one of the major volatiles found in lulo peelings (*19*). Experiments to investigate the role of glucoconjugated **1** as precursor of other C₁₃-compounds present in lulo aroma are under way.

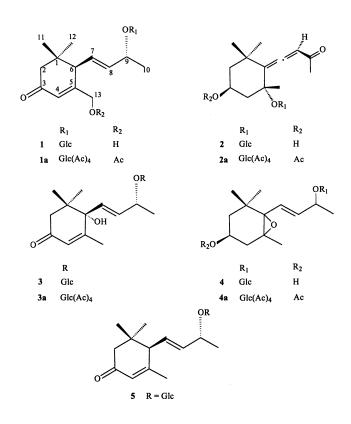


Figure 2. Structures of the glucoconjugated C_{13} -norisoprenoids identified in lulo (Solanum quitoense) leaves.

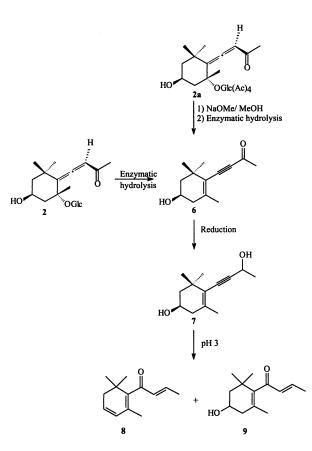


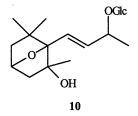
Figure 3. Generation of 3-hydroxy-7,8-dihydro- β -ionone 6, β -damascenone 8, and 3-hydroxy- β -damascone 9 from (3S,5R,8R)-3,5-dihydroxy-6,7-megastigmadien-9-one 5-O- β -D-glucopyranoside 2.

C₁₃-Norisoprenoids in Lulo del Chocó (S. topiro)

The aroma composition (free volatiles) of lulo del Chocó fruit does not exhibit C_{13} -norisoprenoid compounds among the identified volatiles. However, when we performed capillary GC and capillary GC-MS analyses of the glycosidically bound volatile fraction after enzymatic hydrolysis, we found in the pulp as well as in the peelings the following aglycons: 3,6-epoxy-7-

> In Carotenoid-Derived Aroma Compounds; Winterhalter, P., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

megastigmene-5,9-diol and two isomers of vomifoliol (one of them being the 6S,9R-isomer). However, as the above mentioned analyses did not give any information on the sugar moiety of the bound C_{13} -compounds, we decided to perform capillary GC and capillary GC-MS (EI and NCI) analyses of the trifluoroacetylated derivatives of the glycosidic fraction. In this way, we were able to detect and to identify in the fruit pulp the novel 3,6-epoxy-7megastigmene-5,9-diol β -D-glucopyranoside 10, the known (6S,9R)-vomifoliol 9-O-B-D-glucopyranoside 2, and another isomer of vomifoliol 9-O-B-D-glucopyranoside. The identification procedure followed in the structure elucidation of compound 10 is explained in some detailed below. The NCI spectrum of TFA derivative of glycoside 10 shows the ions at m/z 772 [M], 885 [M+TFAO] and 659 [M-TFAO]⁻ clearly indicating a molecular mass of 772. The identity of the sugar was established as a unit of a hexose by the presence of the fragment ions at m/z 677, 563 and 451 in the NCI-MS spectrum and by the ions m/z 319, 291, 205, 193 and 177 in the EI-MS spectrum. The molecular mass of the aglycon was determined as 226 by the difference between the molecular ion and m/z547. Structure determination of the aglycon was assessed by the presence of the characteristic ions at m/z 207, 181, 166, 152, 125, 124 and 109, which were in good agreement with the published spectrum for 3,6-epoxy-7-megastigmene-5,9-diol (20). As far as we know, this is the first time that this glucoside has been found in nature. For more details see ref. (21).



C₁₃-Norisoprenoids in Mammee Apple (*M. americana*)

In Table II free and glycosidically bound compounds as well as C_{13} norisoprenoid glycoconjugates identified in mammee apple fruit are gathered together with the retention indices and the amount of each compound calculated on the basis of the standard added. The free volatile profile was composed, among other important volatiles described in ref. (22), by C_{13} -norisoprenoids (8.1%) with 3-oxo- α -ionone, 3-oxo- α -ionol, 4-hydroxy- β -ionone, 4-oxo- β ionol and 3-oxo-7,8-dihydro- α -ionol being major volatile constituents. Among the glycosidically bound compounds, C_{13} -norisoprenoids represented 17.2 % of the total composition with 3-oxo- α -ionone, 4-hydroxy- β -ionone, 4-oxo- β -ionol, 3-oxo-7,8-dihydro- β -ionol and 4-oxo-7,8-dihydro- β -ionol being major aglycons. Finally, when we examined the TFA glycoside derivatives from *M. americana* by capillary GC and capillary GC-MS (EI, NCI), we could identify 3-oxo- α -ionol 9-O- β -D-glucopyranoside, 4-hydroxy- β -ionone 4-O- β -D-glucopyranoside, 4-oxo- β -ionol 9-O- β -D-glucopyranoside, 3-oxo-7,8-dihydro- β -ionol 9-O- β -D-glucopyranoside and 4-oxo-7,8-dihydro- β -ionol 9-O- β -D-glucopyranoside and 4-oxo-7,8-dihydro- β -ionol 9-O- β -D-glucopyranoside.

Compound	K	I exp	Free	Bound ^a	β-D-
	DB-5	DB-wax			glucopyranoside
β-ionone		1915	+		
β-ionol		1937	+		
3-oxo-α-ionone	1502	2058	++	++	
3-oxo-a-ionol	1686		++		+
4-hydroxy-β-ionone	1697	2533	++	++	+
4-oxo-β-ionol	1703	2544	++	++	+
3-oxo-7,8-dihydro-α- ionone	1723		+	•	
3-oxo-7,8-dihydro-α- ionol	1729	>2600	++	++	+
4-oxo-7,8-dihydro-β- ionol	1738	2585	+	++	+
dehydrovomifoliol	1819	>2600	+	+	
3- ∞o - α -ionol 4-hydroxy- β -ionone 4- ∞o - β -ionol 3- ∞o -7,8-dihydro- α - ionone 3- ∞o -7,8-dihydro- α - ionol 4- ∞o -7,8-dihydro- β - ionol	1686 1697 1703 1723 1729 1729 1738 1819	2533 2544 >2600 2585 >2600	++ ++ + + +	, ++ ++ ++ ++	+ + +

 Table II. C₁₃-Norisoprenoids Identified in Mammee Apple (Mammea

 Americana) Fruit Pulp

^a Glycosidically bound C13-norisoprenoid; $+ = < 100 \ \mu g/kg$; $++ = 100-500 \ \mu g/kg$.

As can be seen from Table II, the glucoconjugated compounds identified in mammee apple could be considered as precursors of most of the C_{13} -norisoprenoids found in free form in the aroma of this fruit.

Acknowledgments.

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Chapter 15

Carotenoid Degradation Products in Paprika Powder

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The color and the characteristic flavor of red paprika powder (*Capsicum annuum* L.) is highly appreciated for savory food products. During processing, treatment and storage of paprika powder numerous degradation and rearrangement products are formed. In this work 29 new carotenoid derived compounds in paprika powder are reported. In addition the presence of 12 previously described carotenoid degradation products was confirmed. Furthermore the influence of individual compounds on the sensorial profile of paprika powder was investigated by means of gas chromatography-olfactometry together with enantioselective gas chromatography.

Capsicum, also called chili or red pepper, is the dried fruit of certain New World relatives of the tomato (*Lycopersicum esculentum*, Solanaceae). Most are the varieties of two species, *Capsicum annuum* (Solanaceae) and the frequently smaller, hotter *Capsicum frutescens* (Solanaceae). One variety of *Capsicum annuum* has become well established in Hungary as sweet bell pepper or paprika (1). In many cuisines paprika is known as spice for its color and flavor. It is applied to food in the form of powder, flakes or oleoresins, which are of growing interest in food industry. The composition of the carotenoid pigments produced in red paprika have been studied thoroughly (2-4). In the following all

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Studies on the volatiles of red pepper, conducted in the early eighties, revealed the presence of β -ionone (I) as an important flavor compound with typical floral notes (5, 6). The importance of β -ionone for the flavor profile of paprika powder was emphasized recently by Zimmermann and Schieberle (7). Parallel to our work, Cremer and Eichner observed a significant increase of β -ionone during heating of paprika powder (8).

Additional publications on volatile compounds in Spanish paprika (9) and paprika oleoresin (10) revealed the presence of other carotenoid degradation products such as dihydroactinidiolide (II). On the one hand these compounds indicate the close relation between color and flavor in dried products like paprika powder. On the other hand carotenoid derived aroma compounds represent paprika-typical sensory properties like, e.g., floral, hay-like and tobacco-like notes. It is well-known that the stability of the main carotenoids depends on the drying conditions and other industrial processes (11). In order to receive an overview about carotenoid derived volatiles in paprika powder several different qualities of paprika powder were analyzed for this work.

EXPERIMENTAL SECTION

Paprika powder. Samples obtained from three different harvest periods (1997-1999) in Hungary were analyzed. Additionally paprika powder of harvest 1999 with and without steam treatment were analyzed.

Sample preparation. For the study of carotenoid degradation products we tested different sample clean-up techniques, for example liquid-liquid extraction, simultaneous distillation extraction (SDE), thermodesorption and extraction with supercritical CO_2 (SFE). A specific procedure starting with a SFE step revealed interesting results both for semi-polar and polar degradation products. Amounts between 300 g and 2000 g of paprika powder were extracted for 4 hours at 40°C and 150 bar with supercritical CO_2 (SITEC System, Switzerland). The obtained extract was subjected to partitioning with two portions of 250 ml distilled water. The next step comprised the extraction of the aqueous phase with dichloromethane and subsequent concentration. All concentrated extracts were analyzed using GC/MS, GC/FTIR and GC/O, as well.

Instrumental analysis. Instrumentation (capillary gas chromatography, spectroscopy) as well as analytical and preparative conditions have been described in a previous publication (12). For chiral separations a fused silica column (25 m x 0,25 mm, film thickness 0,25 μ m) from MEGA capillary columns laboratory (Legnano, Italy) was used. The column was coated with a solution of 30% diacetyl tert. butyl silyl- β -cyclodextrin with 70% OV-1701.

The intensity of the red color is one of the most important parameters that determines the commercial quality of paprika powder. The main carotenoids, which are responsible for the red color spectrum are capsanthin and capsorubin characterized by one and two κ -end groups, respectively.

The red xanthophylls mainly occur as esters with lauric, myristic and palmitic acid. β -Carotene as well as cryptoxanthin, zeaxanthin and violaxanthin are progenitors of red pigments and contribute to the yellow color spectrum. The fatty acids esterifying yellow xanthophylls are linoleic, myristic and palmitic acid. Consequently the red xanthophylls show higher stability than yellow xanthophylls and β-carotene itself at temperatures below 60°C. Above 60°C the situation was found to be inverted (13). The degradation of β -carotene follows different pathways, always with oxygen playing a key role. A welldocumented reaction is photo-oxygenation, induced by singlet oxygen (14). The so called "en"-mechanism and the (2+2) cyclo-addition are important reaction mechanisms. Furthermore, intensive research work revealed the presence of minor constituents with interesting structural properties in varieties of Capsicum annuum. Carotenoids with 3,6-epoxy end groups such as cyclo-violaxanthin, cucurbitaxanthin A, 3,6-epoxyxanthin were found together with (8S)capsochrom, which shows a 5,8-epoxy group (15). Recently the occurrence of 5,6-di-epi-karpoxanthin and 5,6-di-epicapso-karpoxanthin, two derivatives with two and one 3,5,6-trihydroxy-5,6-dihydro β -end groups was reported (16).

Nor Compounds Derived from Acyclic Isoprenoids

In accordance with the work of Guadayol et al. (10), we found 6-methyl-5hepten-2-one (III) and (E)-6-methyl-3,5-heptadien-2-one (IV) as well as geranyl acetone (V) and farnesyl acetone (VI) in the extracts prepared by the above mentioned clean-up procedure. The formation of compound III was also described by Luning et al. in 1994 (17). Geranyl acetone, farnesyl acetone and methyl heptenone are formal degradation products of phytofluene (Figure 1). The formation of methyl heptenone from ξ -carotene and lycopene follows the same scheme. In tomato, as a related fruit, Buttery and Ling suggested an oxidative process for the formation of geranyl acetone, while methyl heptenone (III) also seems to be generated from a glycosidically bound precursor (18). Furthermore (E)-6-methyl-3,5-heptadien-2-one (IV) was found amongst the products of hydrolysis of a tomato glycoside fraction by the same authors. A second pathway to methyl heptadienone was observed by Hohler during SDE treatment of lutein (14).

No.	Compound name	RI (DB-1)	MS-data (m/z, %)
23a	4-hydroxy-2,2,6-	1207	<u>56</u> (15), 83 (100), 57 (58),
	trimethyl-cyclohexanone		69 (45), 41 (39), 82 (38),
	(trans-isomer)		74 (37), 88 (37), 55 (30)
23b	4-hydroxy-2,2,6-	1196	156 (14), 83 (100), 57
	trimethyl-cyclohexanone		(64), 69 (50), 41 (46), 88
	(cis-isomer)		(41), 74 (38), 82 (36), 43
			(32)
29	3,4,4-trimethyl-2-	1016	124 (37), 109 (100), 81
	cyclopentenone		(47), 39 (30), 41 (20), 79
			(16), 53 (13), 40 (10)

 Table I MS-data and retention index data of cyclohexanone and cyclopentenone derivatives

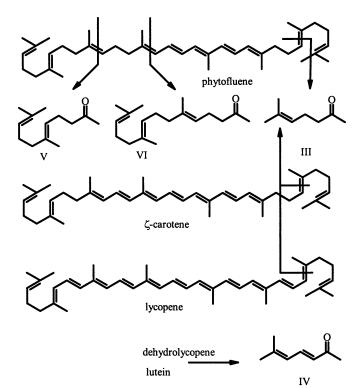


Figure 1. Proposed formation of 6-methyl-5-hepten-2-one (III), (E)-6-methyl-3,5-heptadien-2-one (IV), geranyl acetone (V) and farnesyl acetone (VI) from acyclic isoprenoids (10, 14, 18).

β-Carotene and Cryptoxanthin Derived Cyclic Products

β-Carotene and cryptoxanthin are fairly unstable and give rise to several degradation products, which can be divided into 4 groups according to the number of carbon atoms: C_{13} -, C_{11} , C_{10} - and C_{9} -products.

C₁₃-compounds: β-Ionone (I) and dihydroactinidiolide (II) are known C₁₃norisoprenoids for paprika products (Figure 2). Additionally the presence of αionone (VII) and dihydro-β-ionone (VIII) has been published previously (10). Using GC/MS-data we were able to identify 5,6-epoxy-β-ionone (1), a known degradation product from β-carotene (14). Following the rearrangement of 5,6epoxy-β-ionol proposed by Enzell et al. (19), the corresponding ketone 5,6epoxy-β-ionone can be regarded as a possible precursor for 4-oxygenated C₁₃terpenoids. In this group we identified 4-hydroxy- and 4-oxo-β-ionone, (2) and (3), respectively, in paprika powder (Figure 3). The first compound is a known tobacco constituent (20). Diketone 3 was also obtained by oxidative degradation of canthaxanthin (21).

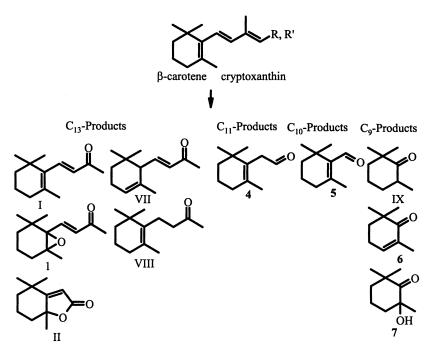


Figure 2. Degradation products from β -carotene and related carotenoids like e.g. cryptoxanthin. Previously described compounds in Roman numerals. New findings in Arabic numerals.

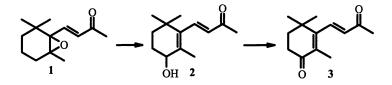


Figure 3. Proposed formation of 4-hydroxy- β -ionone (2) and 4-oxo- β -ionone (3) (modified after 19)

 C_{11} -compounds: The aldehyde 2,6,6-trimethyl-1-cyclohexen-1-acetaldehyde (4), known as β -homocyclocitral is a typical degradation product from β carotene (Figure 2). It has been previously found in red algae (22).

 C_{10} -compounds: 2,6,6-Trimethyl-1-cyclohexen-1-carboxaldehyde (5), also known as β -cyclocitral (Figure 2), is fairly wide spread in nature. It was previously found in paprika oleoresin (10) as well as in tobacco (23).

 C_9 -compounds: In this group we found 2,2,6-trimethylcyclohexanone (IX), which was already described by Guadayol et al. (10) as an ingredient in paprika oleoresin. Additionally we identified the unsaturated derivative 2,6,6-trimethyl-2-cyclohexenone (6) and a corresponding hydroxylated product 2-hydroxy-2,6,6-trimethylcyclohexanone (7) (Figure 2), which was identified in tobacco by Enzell and coworkers (23).

Violaxanthin and Zeaxanthin Derived Products

All carotenoids bearing a hydroxyl group at position 3 of the β -end group are mainly esterified with fatty acids in paprika powder. In comparison to pigments like crypoxanthin, lutein and capsanthin, the carotenoids zeaxanthin and violaxanthin are characterized by one and two 5,6-epoxy-end groups, respectively. Only a few structures derived from violaxanthin and zeaxanthin are currently known for paprika in the literature.

 C_{13} -compounds and C_{11} -compounds: The group of C_{13} -norisoprenoids oxygenated at position 3 comprises 11 new compounds in paprika powder. 3-Hydroxy-dihydro- β -ionone (8) has been found as an aglycone in several fruits of the *Prunus* species (24). A related molecule 3-oxo-7,8-dihydro- α -ionone (9) is not widespread and probably also derived from lutein degradation (Figure 4).

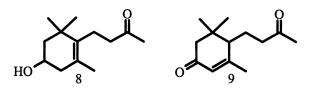


Figure 4. 3-Hydroxy-dihydro- β -ionone (8) and 3-oxo-7,8-dihydro- α -ionone (9)

Violaxanthin is discussed as being a source for 3-hydroxy-5,6-epoxy- β ionone (10) and the corresponding alcohol 3-hydroxy-5,6-epoxy- β -ionol (11). After enzyme hydrolysis of the lyophylized, aqueous fraction we tentatively identified 3-oxo-actinidol, which leads to dehydrololiolide tentatively assigned in the main extract. The C₁₁ product loliolide (12) is another compound in this scheme. It is well-known from tobacco chemistry (25). Regarding the stereochemistry all obtained NMR data for the isolated loliolide were closer to the 3S,5R-isomer than to the 3R,5R-diastereomer. Furthermore 3-hydroxy-5,6epoxy- β -ionone (10) is regarded to be a key compound, which also leads to dehydrovomifoliol (13).

Neugebauer et al. (26) showed that diol 11 can be transformed under acidic conditions into the isomeric 2-(2-butenylidene)-3,3-dimethyl-5(2-oxopropyl) tetrahydrofurans (16a/b) occurring as (2Z)- and (2E)-isomers in stinging nettle (*Urtica dioica* L.). In agreement with Guadayol et al. we identified two naphthalene derivatives (Figure 5). 1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN, X) was described by Humpf and Schreier (27) also as an acid-catalyzed reaction product from diol 11. TDN is a well-known off-flavor compound in wine and characterized by kerosene-like notes. The other derivative 1,2,3,4-tetrahydro-1,1,6-trimethylnaphthalene XI was described in starfruit (*Averrhoa carambola* L.) by Herderich et al. (28).

The next compound 3,4-dehydro- β -ionone (17) was obtained in earlier degradation experiments from lutein (14). It can be regarded as a direct dehydration product of carotenoids with 3-hydroxy- β -end groups and was described in tobacco (23). The second cyclohexadiene derivative 2,3-dehydro- α -ionone (18) consequently has to be regarded as a dehydration product of the 3-hydroxy- α -end group of lutein corroborating our results (Figure 6). For clear evidence, however, degradation studies of lutein have to be conducted.

Our investigations led us to the vomifoliol derivatives vomifoliol (14) and dehydrovomifoliol (13). In quince fruit dehydrovomifoliol as a free norisoprenoid and the glucosidic conjugate of vomifoliol, the so-called roseoside, were identified (29). According to Heckmann and Roberts (30) the most probable theaspirone (15) precursor, however, is considered to be 7,8dihydrovomifoliol, which could not be identified in paprika powder during our study. The break-down of the allenic carotenoid neoxanthin leads to 3-hydroxy- β -damascenone (19) and β -damascenone (20), as well. Both compounds have been identified for the first time in paprika powder. Compound 19 occurs in

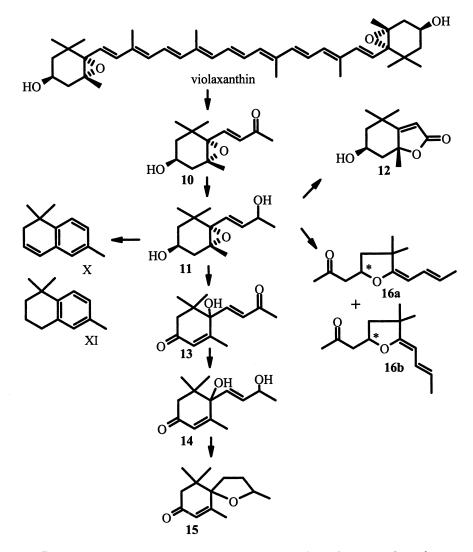


Figure 5. In position 3 oxygenated C_{13} - and C_{12} - degradation products from zeaxanthin, violaxanthin and related carotenoids.

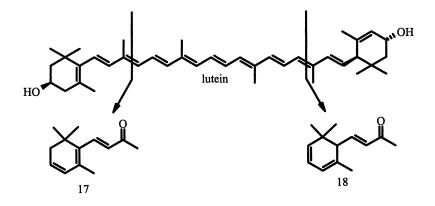


Figure 6. 3,4-Dehydro- β -ionone (17) and 2,3-dehydro- α -ionone (18) as formal degradation products of lutein.

glycosidically bound form in many plants. β -Damascenone (20) is a highly appreciated flavor compound because of its fruity, baked plum-like flavor properties. One of the key intermediates, 3,5-dihydroxy-megastigma-6,7-diene-9-one (grasshopper ketone) has tentatively been identified by us in paprika extracts.

 C_{10} -compounds: 3-Hydroxy- β -cyclocitral (21) and the dehydration product safranal XII are known from saffron flavor chemistry. In saffron the cleavage of the glucoside picrocrocin yields 3-hydroxy- β -cyclocitral, a compound with limited stability, which is transformed to safranal under elimination of water. In 1977 Enzell and coworkers (23) postulated a direct pathway from safranal to 4-methylen-3,5,5-trimethylcyclohexenone (22) following a vinylogous allylic shift (7) (Figure 7).

 C_9 -products: This group is dominated by isophorone derivatives. 4-Oxoisophorone (25) was previously identified in saffron by Straubinger (31) and in tobacco together with 4-hydroxy-2,2,6-trimethyl-2-cyclohexenone (26) by Enzell et al. (23). Tobacco was also the plant material for the first identification of 4-hydroxy-2,2,6-trimethyl-cyclohexanone (23a/b) by Roberts and Rohde (32). We identified both cis- and trans-isomers and the corresponding acetate 24 in paprika powder (Figure 8). The mass spectral data for isomers 23a and 23b are given in Table I.

The pattern of the discussed carotenoid degradation products shows impressive similarities to the natural product chemistry of tobacco. This fact is corroborated by the finding of 2,2-dimethyl-4-hydroxy-6-oxo-heptanoic acid lactone (**28a/b**). According to Isoe et al. (*33*) exhaustive photo-oxygenation of zeaxanthin yields 2,4-dihydroxy-2,6,6-trimethylcyclohexanone. The next step of the postulated formation pathway follows ring cleavage and oxidation to

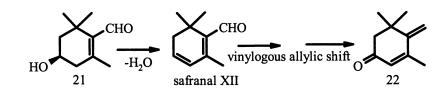


Figure 7. Proposed formation of 3,5,5-trimethylcyclohexenone (22)

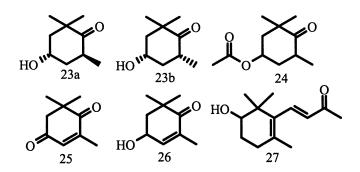


Figure 8. Isophorone related products and 2-hydroxy- β -ionone (27).

hydroxygeronic acid, which is another photo-oxygenation product of zeaxanthin. Finally intramolecular ester formation leads to the above mentioned lactone, which was previously described in tobacco by Lloyd et al. (34). Enantioselective analysis on a chiral GC column revealed racemic distribution of the two enantiomers. The sensory properties of both enantiomers were evaluated using gas chromatography-olfactometry. The compound eluting first was characterized by terpeny, woody notes. The other enantiomer appeared to be more phenolic in odor profile (Figure 9).

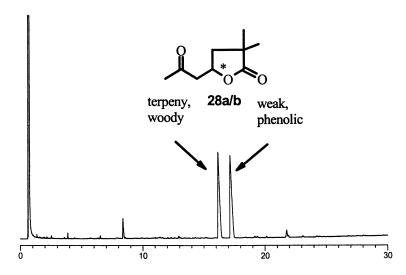


Figure 9. Enantioselective GC-O analysis of 2,2-dimethyl-4-hydroxy-6-oxoheptanoic acid lactone (28a/b) on a diacetyl tert. butyl silyl-β-cyclodextrin column.

In Figure 8 the structure of 2-hydroxy- β -ionone (27) as a rare degradation product is shown. This compound is a known trace constituent of Osmanthus absolute (35). Furthermore the hydroxy ketone has been found in edible algae *Porphyra tenera* (22).

Degradation products of keto carotenoids

The oxidative degradation of capsanthin was described by Philip and Francis (36). Initial steps are the oxidation to capsanthone and the breakdown to β -citraurin, which represents the loss of the κ -end group. Following this pathway the formation of cyclopentenone derivatives have to be expected. In our study, GC/MS analysis of several extracts revealed the presence of 3,4,4-

trimethyl-2-cyclopentenone (29). Compound 29 has previously been identified in aqueous smoke preparations (37) and represents an interesting finding in the context of paprika research. Figure 10 illustrates a possible formation pathway for compound 29, which still must be confirmed by degradation studies with model systems containing capsanthin. For cyclopentenone 29 mass spectral data together with retention index are given in Table I.

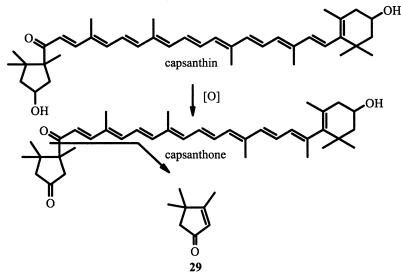


Figure 10. Proposed formation of 3,4,4-trimethyl-2-cyclopentenone (29)

Conclusions

The analysis of non-polar and semi-polar carotenoid degradation products in paprika powder revealed the presence of 29 new molecules and confirmed the presence of 12 previously reported degradation products. The majority of the compounds are known from tobacco flavor chemistry and from a large variety of fruits. With this study, however, it was possible to clarify the strong influence of the carotenoid moiety on the spectrum of volatiles in paprika powder, both representing the most important parameters of quality: color and flavor.

Using GC and GC-O with chiral columns the enantiodifferentiation and sensory evaluation of 2,2-dimethyl-4-hydroxy-6-oxo-heptanoic acid lactone was demonstrated.

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Chapter 16

Flavor Chemistry of Saffron

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Saffron, the dried orange-red stigmas of *Crocus sativus* L. flowers, is widely used in coloring and flavoring of foods. The freshly picked stigmas are nearly odorless, with typical saffron flavor being developed during the drying process. In particular, safranal, the major constituent of the essential oil of saffron, is formed by hydrolysis of the bitter glycoside picrocrocin. In addition to picrocrocin there are numerous other glycosides that may undergo hydrolysis to yield a complex array of compounds that comprise the volatile profile of saffron. Safranal and these other hydrolysis products may undergo further degradation to yield additional volatile compounds. In this report, recent developments in the chemistry and analysis of saffron flavor will be reviewed.

The spice saffron is derived from the flowers of *Crocus sativus*, Linné, (synonym *Saffran officinarum*), a perennial herb of the family Iridaceae (1). Saffron derives its name from the Arabic word za'faram, which means yellow. Other names for saffron include safran (French, German), azafran (Spanish), and zafferano (Italian) (2). The origins of saffron can be traced back to ancient Greece, Asia Minor, and Persia (1). Sometime later, cultivation and use of saffron spread eastward to northern India and China. Saffron was introduced to Spain by the Arabs in the tenth century AD. Currently, major saffron producing regions include Iran, Spain, and India.

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The dried spice of commerce is composed of the reddish-orange filaments of the dried tripartite stigma, with attached style, of about 2 to 3 cm in length (1). Each crocus flower produces three stigmas and, in general, it requires approximately 70,000 stigmas to produce one kilogram of spice. Saffron is considered to be the most expensive spice and has a market price of US\$ 1,000-2,000 per kilogram depending on quality. Worldwide production of saffron is approximately 50,000 kilograms (3). Throughout history, saffron has found various uses, such as a textile dye, drug, spice, and for ceremonial purposes. Nowadays, saffron is used almost exclusively for its flavoring and coloring properties. General reviews on the history, botany, production and commerce, and chemistry of saffron are available (1-5).

Sensory Properties of Saffron

From a culinary standpoint, saffron provides coloring properties, bitter taste, and a distinctive aroma described as sweet, floral, fatty, savory, spicy and herbaceous (2, 6). Descriptive sensory analysis has been used to identify and quantify the individual aroma attributes of mongra saffron (from Kashmir, India) (7). The flavor profile depicted in Figure 1 is for a saffron sample considered to have a typical strong, yet balanced aroma profile consisting of distinct floral, sweet and spicy aroma notes, with minimal contribution by harsh/acrid and barky notes. Harsh/acrid and barky notes were found to have a lasting impression when the saffron aroma was evaluated on a paper strip (7). It was noted that inferior quality saffron would have overall lower intensity scores with dominant harsh and acrid end notes (7).

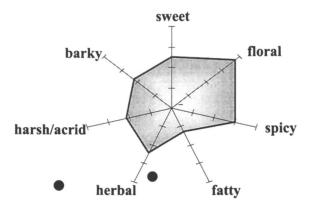


Figure 1. Sensory aroma profile of select grade mongra saffron (7).

Major Secondary Metabolites of Saffron

The major components (secondary metabolites) responsible for the flavor and color of saffron are *cis*- and *trans*-crocins, picrocrocin and safranal (8-13). Crocin, the digentiobioside of crocetin (2,6,11,15-tetramethylhexadeca-2,4,6,8, 10,12,14-heptaenedioic acid), and other crocetin glycosides - collectively referred to as the 'crocins' are the principal yellow-red pigments of saffron (14-18). Glucose, gentiobiose, and neopolitanose, as esters of crocetin, comprise the carbohydrate residues of the crocins (18). At least seven types of crocins exist in saffron with crocin, the digentiobiose ester of crocetin, being the most abundant (18). In addition to the crocins, minor amounts of the C₄₀-carotenoids phytoene, phytofluene, tetrahydro-lycopene, β -carotene, and zeaxanthin are found in saffron (19). Other minor components include flavonoids with potential biological activity, such as glycoconjugates of kaempferol (5,20).

Because of the presence of C_{40} -carotenoids and absence of C_{20} -carotenoids (e.g. lycopersene, phytoene, phytofluene, and δ -carotene) it has been suggested that synthesis of crocetin, the C_{20} -aglycone of the main pigments, occurs via oxidative degradation of the C_{40} -carotenoid zeaxanthin by a hypothetical 7,8carotenase enzyme (19). The proposed mechanism involves the cleavage of zeaxanthin along the polyene chain of the double bonds directly adjacent to the ring systems to yield crocin (1 mol) and picrocrocin (2 mol) (Figure 2) (19). This pathway is supported by occurrence of picrocrocin having the same stereochemical configuration (4*R*) as zeaxanthin at the hydroxylated carbon (21).

The odorless and colorless picrocrocin is responsible for the bitter taste of saffron and is the precursor of safranal, the major volatile component of saffron (9,19,21-23). Fresh stigmas are virtually odorless and the characteristic aroma of saffron is generated during drying by enzymatic and/or thermal degradation (4-β-D-glucopyranosyloxy-2,6,6-trimethyl-1-cyclohexen-1of picrocrocin carboxaldehyde) to form glucose and 4R-hydroxy-β-cyclocitral (12,21,29). 4R-Hydroxy- β -cyclocitral can readily undergo dehydration to form safranal (2,6,6trimethyl-1,3-cyclohexadien-1-carboxyaldehyde) (Figure 3) (12,21). Alternatively, under extreme conditions, such as high temperatures or acidic or basic conditions, picrocrocin may undergo dehydration to form safranal directly (Figure 3) (19). Safranal was first identified by Kuhn and Winterstein (22) and synthesized shortly thereafter by Kuhn and Wendt (24). Today, safranal is offered commercially as a flavor chemical.

Volatile Components of Saffron

Over 60 volatile compounds have been identified in saffron (Table I), with many more having yet to be identified. As stated earlier, Kuhn and Winterstein

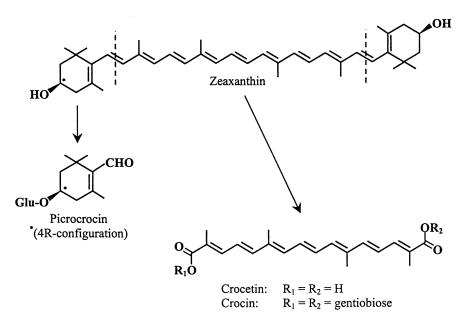


Figure 2. Proposed pathway for the biodegradation of zeaxanthin into color (crocins) and flavor (picrocrocin) principles of saffron

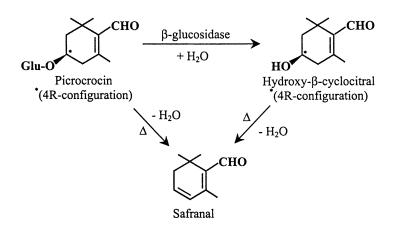


Figure 3. Degradation of picrocrocin to hydroxy- β -cyclocitral and safranal

(22) first elucidated the structure of safranal (1) in the early 1930's. Since then, safranal has been generally regarded as the major aroma component of saffron and has been found to comprise as much as 60 to 70% of the essential oil content (25-30). However, there are numerous other volatile compounds that contribute to saffron aroma.

Zarghami and Heinz (31,32) conducted the first in-depth studies on the volatile components saffron in the early 1970's. In these studies, saffron was extracted with diethyl ether and the volatile constituents separated by gas chromatography. In the first report by these researchers (31), eight volatile constituents were identified including safranal (comprising 47 % of the total peak area), isophorone (3,5,5-trimethyl-2-cyclohexen-1-one; 2), 4-hydroxy-3,5,5-trimethyl-2-cyclohexen-1-one (3), 2,6,6-trimethylcyclohexane-1,4-dione 2,6,6-trimethyl-2-cyclohexen-1,4-dione (5), 2-hydroxy-3,5,5-trimethyl-2-(4), cyclohexen-1,4-dione (6), 4-hydroxy-2,6,6-trimethyl-1-cyclohexen-1-carboxaldehyde (7), and 4-hydroxy-2,6,6-trimethyl-3-oxo-1,4-cyclohexadien-1-carboxaldehyde (8). Relative abundances of these compounds on the basis of safranal (assigned 100) were 2 (3.94), 3 (12.80), 4 (3.28), 5 (2.47), 6 (2.47), 7 (29.40), and 8 (12.80). In a subsequent report, Zarghami and Heinz (32) reported on, in addition to compounds nos. 1-8, the identification of 2,3-epoxy-4-(hydroxymethylene)-3,5,5-trimethyl-cyclohexanone (9), 3-hydroxy-2,6,6-trimethyl-4-oxo-2-cyclohexen-1-carboxaldehyde (10), 2-methylene-6,6-dimethyl-3-cyclohexen-1-carboxaldehyde 3,5,5-trimethyl-4-methylene-2-cyclo-(11), hexene-1-one (12), 2,6,6-trimethyl-3-oxo-1,4-cyclohexadien-1-carboxaldehyde (13) and several other volatile compounds (2-phenyl ethanol, naphthalene, and 2-butenoic acid lactone).

It was suggested by Zarghami and Heinz (31,32) that isophorone-related compounds may be formed through oxidation and decarboxylation of safranal followed by the further oxidation and isomerization of compound 3. However, because both oxidized and reduced isophorone-related compounds were present in saffron, they further proposed these compounds could have been formed enzymatically.

Rödel and Petrzika (26) used simultaneous distillation – extraction with diethyl ether/pentane (2:1) as the solvent to isolate the volatile constituents of saffron. Safranal (1) (60 % of the total area) was most abundant with 36 other compounds being tentatively identified on the basis of GC-MS analysis. Compounds nos. 14-35 were newly identified as saffron volatile constituents. Results of sniffing of the gas chromatographic effluent (olfactometry) demonstrated that safranal was a key aroma component of saffron; however, many additional odor-active compounds were detected as well. Many of the identified compounds were structurally similar to safranal and it has been proposed by several researchers that these compounds may be formed via oxidation of safranal (31,32). Rödel and Petrzika (26) further proposed that compounds with a partially unsaturated C_4 side chain (in C1 position) could be

formed by cleavage of zeaxanthin along the polyene chain at double bond positions removed from the ionone ring, possibly by stepwise terminal degradation.

Tarantillis et al. (29) evaluated three isolation techniques for analysis of saffron volatile compounds. More compounds were isolated by steam distillation (SD, 18 compounds) than by either micro-simultaneous distillationsolvent extraction (SDE, 13 compounds) or vacuum headspace trapping (HS, 8 compounds). The greater number of compounds isolated by SD and SDE was thought to be due these techniques being more efficient for isolation of components with higher boiling points and possibly due to formation of artifacts due to oxidation/thermal degradation of saffron carotenoids during the isolation An example of such a degradation product is 3,7-dimethyl-1,6procedure. octadiene (36). Safranal (1) was the major component, comprising nearly 70% of total peak area of all components, followed by isophorone (2) (~14%), 3,5,5trimethyl-3-cyclohexen-1-one (18) (~5%), 2,6,6-trimethyl-2-cyclohexen-1,4dione (5) (~4%), and 2,6,6-trimethyl-1,4-cyclo-hexadiene-1-carboxaldehyde (37) (~3%). Several compounds were identified for the first time in saffron (36-**43**). Recently, Winterhalter and Straubinger (5) reported on the volatile profile of Greek saffron (*electus pulvis*). Volatile components were isolated by direct solvent extraction with ether using a Soxhlet apparatus and analyzed by GC-MS. Most of the identified components had been previously reported in saffron (e.g. nos. 1-9, 14); however, three newly identified constituents were detected. These included 2,6,6-trimethyl-2-cyclohexan-1-one (53), 4-hydroxymethyl-3,5,5-trimethyl-2-cyclohexen-1-one (54), and 3-hydroxy-α-ionone (55).

Aroma-active components of saffron

In a study conducted in our laboratory, we isolated the volatile constituents of Spanish saffron (Mancha Superior) by two extraction methods, direct solvent extraction (DE) and atmospheric simultaneous steam distillation-solvent these extraction (SDE), and subsequently analyzed extracts by gas chromatography-olfactometry (GCO) and GC-MS (6). Aroma extract dilution analysis (AEDA) was used to indicate the predominant aroma-active components in each type of extract. AEDA is a method that involves the GCO evaluation of a stepwise dilution series of a volatile extract (33) and allows for the determination of flavor dilution (FD)- factors. A FD-factor is the highest dilution at which a specific aroma compound can be detected by GCO, and is used to arrange or rank the aroma-active compounds according to their odor potency in the extract.

DE and SDE resulted in extracts having different volatile profiles, with respect to levels of major components as well as in numbers and types of volatiles identified. In general, more thermal decomposition products were identified in SDE extracts. This was evident by the presence the sugar breakdown products, 2-furan-carboxaldehyde and 5-methylfurancarboxaldehyde in SDE extracts. SDE extracts also contained higher levels of four megastigmatriene isomers (44 and 45) which have reported to be carotenoid decomposition products (34).

In DE and SDE extracts, safranal (1) was the most abundant volatile compound, followed by isophorone (2) and 2,6,6-trimethyl-2-cyclohexene-1,4dione (5). Other major volatile compounds included 2,6,6-trimethylcyclohexane-1,4-dione (4), 2-hydroxy-3,5,5-trimethyl-2-cyclohexen-1,4-dione (6), 2-4-hydroxy-2,6,6-trimethyl-3-oxo-1,4-cyclohexadien-1-carboxphenylethanol, aldehyde (8), and 2-methylene-6,6-dimethyl-3-cyclohexen-1-carboxaldehyde (11). These findings were in general agreement with those of previous studies (26,29,31). Many additional components were identified for the first time in saffron. These included compounds nos. 44-52 in Table I, those marked with an asterisk in Table II, and several others (3-hydroxy-2-butanone; 2-2-methylpropanoic furancarboxaldehyde; acid; 5-methyl-2-furancarboxaldehyde; 2,3-butanediol; sulfinylbismethane; dihydro-2(3H)-furanone; 2phenylacetic acid; hexanoic acid; benzenemethanol; sulfonylbismethane).

Results of GCO and AEDA revealed that numerous volatile components contribute to saffron aroma. DE and SDE extracts had distinctive saffron-like aromas but were slightly different from one another. DE extracts had sweet, spicy, and floral notes and were most similar to the dried saffron; whereas SDE extracts had nutty, cooked rice- and hay-like aroma notes, but retained definite saffron-like characteristics. A combined total of 25 aroma-active compounds were detected in DE and SDE extracts by GCO and AEDA. Eighteen of these were common to both extracts. Many of these 25 compounds can be roughly grouped using the sensory attribute terms (sweet, floral, spicy, fatty, herbal, harsh/acrid, and barky) developed by Narasimhan et al. (7) (Table II; see also Figure 1). It is evident that many compounds could be grouped under more than one term, while some compounds do not fall under any of these terms.

2-Hydroxy-4,4,6-trimethyl-2,5-cyclohexadien-1-one (14) was detected at the highest \log_3 FD-factor, followed closely by safranal (1). This finding was somewhat surprising since in previous reports safranal has been regarded as the character-impact aroma component of saffron. This might be explained by the fact that compound no. 14 has been typically found at a much lower levels, approximately 10-20-fold less, than safranal (6). In addition to these compounds, 3,5,5-trimethyl-3-cyclohexen-1-one (18) and several unidentified, but presumably carotenoid-derived, compounds contributed saffron-like aroma notes and had moderately high \log_3 FD-factors (Table II).

Many additional compounds contributing to the overall aroma of saffron were generated through lipid oxidation (e.g. 1-octen-3-one, (E,Z)-2,6-nonadienal, and (E,E)-2,4-decadienal), *Maillard* reaction/*Strecker* degradation (e.g. 2,3-butanedione, 2-acetyl-1-pyrroline, 3-(methylthio)propanal, 3-methylbutanoic acid), and hydrolysis of non-carotenoid glycoside precursors

No.	Compound	Structure	Reference
1	2,6,6-trimethyl-1,3-cyclohexadien-1- carboxyaldehyde (safranal)	СНО	5, 6, 10- 12, 26-33
2	3,5,5-trimethyl-2-cyclohexen-1-one (isophorone)	₩ L	5, 6, 27, 31-33
3	4-hydroxy-3,5,5-trimethyl-2- cyclohexen-1-one	OH U	5,6, 27, 31-33
4	2,2,6-trimethylcyclohexane-1,4-dione (isomers)	↓ ↓	5,6,27, 30-33
5	2,6,6-trimethyl-2-cyclohexen-1,4- dione		5, 6, 27, 30-33
6	2-hydroxy-3,5,5-trimethyl-2-cyclo- hexen-1,4-dione	Он	5, 6, 27, 31-33
7	4-hydroxy-2,6,6-trimethyl-1-cyclo- hexen-1-carboxaldehyde (hydroxy-β- cyclocitral; 4-β-hydroxysafranal)	CHO OH	5, 27, 29, 32, 33
8	4-hydroxy-2,6,6-trimethyl-3-oxo-1,4- cyclohexadien-1-carboxaldehyde	CHO CHO OH	5, 27, 30, 32, 33
9	2,3-epoxy-4-(hydroxymethylene)- 3,5,5-trimethylcyclohexanone	CH ² OH	5, 33

Table I. Volatile Carotenoid-Derived Components of Saffron

Continued on next page.

No.	Compound	Structure	Reference
10	3-hydroxy-2,6,6-trimethyl-4-oxo-2- cyclohexen-1-carboxaldehyde	Сно	5,32
11	2-methylene-6,6-dimethyl-3- cyclohexen-1-carboxaldehyde	CH0 CH1	5,6, 27, 31, 32
12	3,5,5-trimethyl-4-methylene-2- cyclohexen-1-one	V	27, 30, 32
13	2,6,6-trimethyl-3-oxo-1,4-cyclo- hexadien-1-carboxyaldehyde	СНО	27, 32
14	2-hydroxy-4,4,6-trimethyl-2,5- cyclohexadien-1-one	он	5,6, 27, 30, 31
15	2,4,6-trimethylbenzaldehyde	CHO	6, 27
16	4-hydroxy-2,6,6-trimethyl-3-oxo-1- cyclohexen-1-carboxaldehyde	СНО	6, 27
17	5,5-dimethyl-2-cyclohexen-1,4-dione		27, 30
18	3,5,5-trimethyl-3-cyclohexen-1-one	₹Ţ	6, 27, 30

Table I continued

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Table 1 continued					
No.	Compound	Structure	Reference		
19	2,6,6-trimethyl-3-oxo-1-cyclohexen- 1-carboxaldehyde	СНО	6, 27, 30		
20	3,3-dimethyl-1-cyclohexene	\sim	27		
21	2,2-dimethyl-4-oxocyclohexan-1- carboxaldehyde	CHO 0	6, 27		
22	2-hydroxy-3,5,5-trimethyl-4- methylene-2-cyclohexen-1-one	CH ₂ OH	27		
23	2,4,6,6-tetramethyl-1-cyclohexen-1- carboxaldehyde	CHO	6, 27		
24	2-hydroxy-3-methyl-5,6,7,8- tetrahydro-1,4-naphthochinon	С	27		
25	2,3-dihydro-1,4-naphthochinon	ОН	27		
26	3-(but-1-enyl)-2,4,4-trimethyl-2- cyclohexen-1-ol	CH=CHCH ₂ CH,	27		
27	2,6,6-trimethyl-5-oxo-1,3- cyclohexadien-1-carboxaldehyde	CHO	6, 27		
28	methyl 2,6-dimethylbenzoate	COOCH ³	27		

Table	I	continued
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Continued on next page.

No.	Compound	Structure	Reference
29	2,6,6-trimethyl-1,3-cyclohexadien-1- carboxylic acid	Соон	27, 30
30	2,2-dimethylcyclohexane-1- carboxaldehyde	СНО	27
31	1-(but-1-enyl)-2,6,6-trimethyl- cyclohexa-1,3-diene (isomers)	CH=CHCH2CH3	27
32	3-(but-1enyl)-2,4,4-trimethylcyclo- hexan-1-ol	CH=CHCH ₂ CH ₃	27
33	5-(buta-1,3-dienyl)-4,4,6-trimethyl- 1,5-cyclohexadien-1-ol	CH=CHCH=CH ₂	6, 27
34	1,3,3-trimethyl-2-(3-oxobut-1-enyl)- 1-cyclohexene (β-ionone)	CH=CHCCH3	27, 30
35	2,4,4-trimethyl-3-(3-oxobut-1-enyl)- cyclohexan-1-ol (2 isomers)	CH=CHCCH ₃	27
36	3,7-dimethyl-1,6-octadiene		30
37	2,6,6-trimethyl-1,4-cyclohexadien-1- carboxaldehyde	СНО	30
38	2-hydroxy-5-cyclohexen-1,4-dione	но	30
39	3,3,4,5-tetramethylcyclohexan-1-one	\downarrow	30

Table I continued

No.	Compound	Structure	Reference
40	4,6,6-trimethyldicyclo-[3.1.1]hept-3- en-2-one	Ŷ	30
41	4-hydroxy-2,6,6-trimethyl-3-oxo- cyclohexan-1-carboxaldehyde	CHO OH	30
42	4-(2,2,6-trimethylcyclohexan-1-yl)-3- buten-2-one (dihydro-β-ionone)	CH=CHCCH,	30
43	2,4,4-trimethyl-3-(3-oxo-1-butenyl)- 2-cyclohexen-1-ol	сн=снёсн, снорн	30
44	megastigma-7,9,13-triene (isomers)	CH=CHCH=CH ₂	6
45	Megastigma-4,6,8-triene (isomers)	СНСН=СНСН, СНО	6
46	2,6,6-trimethyl-4-oxo-2-cyclohexen- 1-carboxaldehyde	₩ V	6
47	4-hydroxy-2,6,6-trimethyl-2- cyclohexen-1-one	P OH	б
48	2-hydroxy-3,5,5-trimethyl-2- cyclohexen-1-one	Сон	6
49	2,6,6-trimethyl-2,4-cycloheptadien-1- one	X	6

Table I continued

Continued on next page.

No.	Compound	Structure	Reference
50	5-tert-butyl-1,3-cyclopentadiene	C(CH ₃) ₃	6
51	geraniol	Сн2он	б
52	6,10-dimethylundeca-5,9-dien-2-one (geranyl acetone)	hand have	6
53	2,6,6-trimethyl-2-cyclohexen-1-one	Y III	5
54	4-hydroxymethyl-3,5,5-trimethyl-2- cyclohexen-1-one	CH ₂ OH	5
55	3,5,5-trimethyl-4-(3-hydroxy-1- butenyl)-1-cyclohexen-1-ol (3-hydroxy-α-ionone)	он сн=снснсн, он	5

Table I continued

••••••••••••••••••••••••••••••••••••••		Average	
Compound	Aroma Description ^b	log ₃ FD-	-factor ^c
		SDE ^d	DE
	Sweet		
2,3-butanedione*	buttery, cream cheese	<1	<1
4-hydroxy-2,5-dimethyl-3(2H)-	cotton candy,	nd ^f	<1
furanone	strawberries		
	Floral		
3,5,5-trimethyl-3-cyclohexen-1- one (18)	saffron, floral, hay	1.33	<1
linalool	floral, honeysuckle	1.67	<1
2-phenylethanol	floral, rose	<1	1.33
unknown	floral, rose, saffron	3.17	<1
	Spicy		
unknown	saffron, dried hay	2.0	1.17
2,6,6-trimethyl-1,3-cyclohexadien-	saffron, tea	5.33	4.0
1-carboxaldehyde (safranal) (1)	54)), 611, 104	0.00	
unknown	saffron, dried hay	2.17	4.5
2-hydroxy-4,4,6-trimethyl-2,5-	saffron, stale, dried hay	5.5	5.5
cyclohexadien-1-one (14)			
•	Fatty		
(E,Z)-2,6-nonadienal*	sweet, cucumber	1.0	<1
unknown	stale, soapy	nd	<1
(E,E)-2,4-decadienal [*]	fatty, fried fat	1.0	1.0
(_,_, _, _,	Herbal		
1-octen-3-one [*]	mushroom earthy	2.0	1.17
unknown	cooked rice, baking bread	3.5	2.0
3-methylbutanoic acid [*]	rotten, sour, dried fruit	2.0	1.67
unknown	fruity, stale	<1	1.33
undrown	Harsh/Acrid	•	1100
unknown	plastic water bottle	<1	nd
acetic acid [*]	vinegar, acidic	nd	<1
accile acid	-	nu	~1
	Barky	1 22	~1
unknown	stale, bitter, dried hay	1.33	<1
unknown	stale, bitter	1.33	nd
	Other		
unknown	sour, dark chocolate	<1	<1
2-acetyl-1-pyrroline*	nutty, popcorn	2.83	<1
3-(methylthio)propanal	baked potato	1.67	nd
unknown	green onion	1.83	1.33

Table II. Aroma-Active Components of 'Mancha Superior' Saffron^a

^aAdapted from ref. 6. ^bAroma description assigned during gas chromatographyolfactometry (GCO). ^cAverage log3FD-factor (n = 6). ^dSDE, atmospheric simultaneous distillation-solvent extraction. ^eDE, direct solvent extraction. ^fnd, not detected during GCO. ^{*}Indicates compound was not previously identified in saffron. (e.g., linalool, benzenemethanol, 2-phenylethanol). The presence of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone in saffron could be explained by either *Maillard* reaction or possibly by its release from a glycoside precursor. Based on the above findings, it can be concluded that in addition to the carotenoid-derived compounds many other non-carotenoid compounds impact the aroma of saffron.

Glycosides as Aroma Precursors

Recent studies conducted by Winterhalter and coworkers (5,35-37) have demonstrated that in addition to picrocrocin there exists numerous other glycosidic aroma precursors in saffron. Gas chromatographic screening of enzymatically (glycosidase) liberated aglycons of Greek saffron revealed that 3, 6-9, 14, 16 (Table I), as well as 2-phenylethanol, exist in compounds nos. glycosidically bound form (5). Glycosides were isolated using multilayer coil countercurrent chromatography and high performance liquid chromatography and then characterized using spectroscopic methods (35-37). Initially, four glycosides were reported (35) (nos. cf. Figure 4): (4R)-4-hydroxy-3,5,5trimethyl-2-cyclohexenone O-β-D-glucopyranoside (1a), (4S)-4-hydroxy-3,5,5trimethyl-2-cyclohexenone $O-\beta$ -D-glucopyranoside (2a), (4S)-4-(hydroxymethyl)-3,5,5-trimethyl-2-cyclohexenone O-β-D-glucopyranoside (3a), and 2methyl-6-oxohepta-2,4-dienoic acid (4a). Additional glycosides were later reported (36,37): (4R)-4-hydroxy-2,6,6-trimethyl-cyclo-1-hexene-carboxaldehyde O- β -D-gentiobioside (5a), (4R)-4-hydroxy-2,6,6-trimethyl-1-cyclohexenecarboxylic acid O-\beta-D-glycopyranoside (6a), 6-hydroxy-3-(hydroxymethyl)-2,4,4-trimethyl-2,5-cyclohexadienone 6-O-β-D-glucopyranoside (7a), (2Z)-3-methyl-2-pentenedioic acid 1-[1-(2,4,4-trimethyl-3,6-dioxocyclohexenyloxy)-O-β-D-glucopyranosid-6-yl] ester (8a), 5-hydroxy-7,7-dimethyl-4,5,6,7tetrahydro-3H-isobenzofuran-1-one O-β-D-glucopyranoside (9a), (1R,5S,6R)-5-(hydroxymethyl)-4,4,6-trimethyl-7-oxabiocylco[4.1.0]heptan-2-one O-β-D-glucopyranoside (10a), (1R)-3,5,5-trimethyl-3-cyclohexen-1-ol O-β-D-glucopyranoside (11a), roseoside (12a), and glucosides of 4-hydroxydihydrofuran-2one (two isomers), 2-phenylethanol, and benzenemethanol. It was proposed that the above glycoconjugates would undergo hydrolysis and dehydration to yield known saffron volatile components (5, 35-37).

The existence of the above glycosides, in addition to picrocrocin, suggests that numerous pathways may be involved in the biodegradation of zeaxanthin (Figure 4) (5, 36).

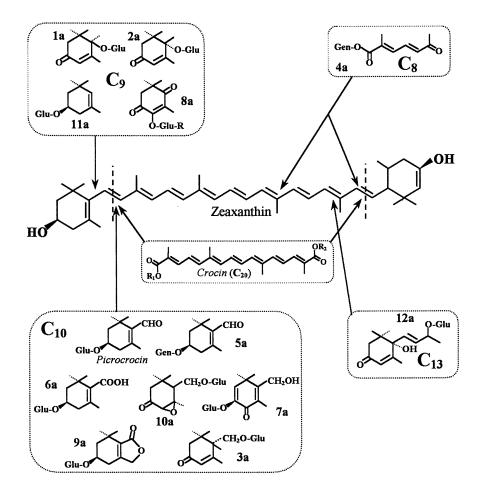


Figure 4. Proposed degradation of zeaxathan in the formation of $C_{\$}$, C_{9} , C_{10} , C_{13} and C_{20} glycosides (Adapted from ref. 5. Copyright 2000 Marcel Dekker, Inc.)

Several factors are known to influence the formation and retention of secondary metabolites in saffron, namely cultivation/harvesting practices and postharvest/ storage conditions. During cultivation of *C. sativus*, the crocins and picrocrocin increase from the period before bloom and reach a maximum during full bloom period (28,38). Therefore, a greater yield of saffron is obtained from flowers at full bloom (42% more than at first appearance of buds) (28). Due to the high cost of cultivation and harvesting of saffron, alternative methods of production have been investigated. It is possible to develop stigma-like structures *in vitro* via tissue culture of excised young stigmas and ovaries of *C. sativus* (23) or from callus tissue (23,39). Furthermore, under certain conditions it is possible to induce the production of crocin, picrocrocin (23,39) and the drying (and heating) of these stigma-like structures was found to result in formation of safranal and hydroxy- β -cyclocitral (23).

The shelf-life and overall quality of saffron are primarily dependent on the disposition of the three major secondary metabolites; the yellow pigment 'crocin', the bitter-tasting glucoside 'picrocrocin' and the aroma-impact compound 'safranal'. The levels of these components are greatly affected by post harvest handling conditions, especially temperature during drying (28). It is known that the characteristic aroma of saffron is produced during drying of the freshly harvested stigmas. During this process the bitter glycoside picrocrocin is hydrolyzed to produce safranal (23). The two drying techniques in general use are sun-drying and toasting over an open fire (23). In India, saffron is produced by sun-drying of stigmas for 3-5 days to reduce moisture content to 10-12%. Alternatively, during bumper flower harvest, the whole flowers are sun-dried and stigmas later picked from the dried material (28). In Spain (La Mancha region), saffron is processed by slow dehydration over hot ashes of a wood-fire until the weight has been reduced to approximately 1/5 of the original wet weight (27). This process gives superior saffron with the desired coloring and flavoring potency. In general, shorter drying periods at controlled temperature results in a saffron of superior quality due to retention of crocins, while allowing for the desired level of degradation of picrocrocin to safranal.

A detailed investigation of drying techniques on quality of Indian saffron was conducted by Raina et al. (28). Their results showed that, in general, drying at low temperatures (below 30°C) for prolonged periods (27-53 h) gave poorer quality saffron due to significant loss of crocins, presumably due to biodegradation (enzymatic hydrolysis). Use of higher temperatures required considerably less time (2-4 h) but also resulted in significant degradation of pigments (measured as crocins). Efficient cross flow and vacuum oven processes resulted in excellent retention of pigments but poor flavor development due to formation of 4-hydroxy- β -cyclocitral as main volatile (50 % or more) instead of safranal. With respect to highest pigment retention and overall apperance, optimum temperature range was $35-50^{\circ}$ C for 4 - 7 h regardless of drying method employed (oven, vacuum oven, cross-flow dryer or solar dryer). Based on practically and overall saffron quality (color and flavor) it was recommended that drying be conducted at $40\pm5^{\circ}$ C using either a solar cabinet (6.3 h) or an oven.

The kinetics of saffron carotenoid degradation under varying storage conditions has been reported (40,41). Degradation of saffron carotenoids, specifically the crocins, follows first-order reaction kinetics. The degradation rate is greatly influenced by temperature, water activity, pH, light, and oxygen (12,28,38,40,41). It has been reported that intermediate water activity ($a_w \sim 0.43-0.53$) favors aroma formation by release of volatile aglycons, such as safranal by hydrolysis of picrocrocin (41). Meanwhile, within this water activity range the rate of breakdown of the crocins appears to be reasonably low. The general practice is to maintain a moisture level of approximately 5%, since degradation of the crocins is related to moisture content (28).

Quality Assurance and Detection of Adulteration

Because of its high market price saffron has been adulterated throughout its history. Furthermore, saffron from different geographic locations and produced by different processing methods varies in quality with respect to color and flavor. Many studies have focused on saffron quality determination and detection of adulteration. Grading of saffron is done visually and quality control standards of the International Organization for Standardization (ISO) (42) also are applied to saffron traded on the international market. These include certain microscopic and chemical requirements. Classification is done by spectrophotometry in the UV-visible range, where three absorption maxima are observed at 257 nm (picrocrocin), 330 nm (safranal), and 440 nm (crocins). However, considerable spectral interference or overlap occurs between the various saffron components (11, 12) and use of an aqueous extract may not be suitable for all saffron constituents (30). That is, this assay may not give an accurate representation of safranal content due to the limited solubility of safranal in water and the fact that other saffron constituents (e.g., cis-isomers of crocin) absorb light at the same wavelength (330 nm) as safranal (27). Several researchers have proposed the use of alternative methods for saffron quality determination and for detection of adulturation. These methods include high performance liquid chromatography (HPLC) (9-13,17), gas chromatography (7,26,28,30), sensory evaluation (7), and multivariate analysis (43).

High performance liquid chromatography has been successfully applied for the determination of crocin, picrocrocin, and safranal (9-13,17). Sujata et al. (10) compared thin-layer chromatography (TLC), HPLC, and GC for the determination of saffron quality. Results from TLC and HPLC were comparable for the determination of crocin and crocetins, picrocrocin and safranal. GC was only suitable for determination of saffron. In general, due to its simplicity, sensitivity and ability to measure several important saffron components, HPLC was regarded as the best overall method for detection of adulteration and quality determination.

Gas chromatographic methods have been developed for the determination of safranal as well as for detection of adulteration (7,25,27,30). Alonso et al. (30) employed purge-and-trap (thermal desorption)–GC-MS to determine saffron volatile constituents in an effort to detect adulteration. Based on analysis of 252 different saffron samples (Spanish producers), the technique was demonstrated to be effective for the detection of the presence of "false saffron" (*Carthamus tinctorius L.*) or the addition of synthetic (artificial) safranal (by detection of a minor component β -cyclocitral (30)).

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Chapter 17

The Formation of β-Damascenone in Wine

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Proposed mechanisms for the formation of β -damascenone from the carotenoid neoxanthin in nature and in food products are discussed. Glycoconjugation of multifunctional precursors is likely to be an important factor in determining yields of β -damascenone from these precursors.

β-Damascenone was isolated from Bulgarian rose oil by Demole and coworkers (1) and first identified in grape and wine by Schreier and Drawert (2). β-Damascenone has a very low odor threshold of 2 ng/L in water (3). More recently, an odor threshold of 50 ng/L in 10 % alcohol has been determined (4). The C₁₃-ketone exhibits a complex aroma which has been described as honey-like, flowery and ionone-like which can vary depending on concentration (5,6). β-Damascenone belongs to a family called the rose ketones, whose importance has seen a ten ton annual production of these compounds in the flavor and fragrance industry (7). In a recent review, it is reported that βdamascenone has been identified in 61 different edible materials, and that since the discovery of the rose ketones, about 300 different patent and scientific publications have appeared on this subject (8).

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A mode of β -damascenone formation was first proposed by Isoe et al. (9) and Ohloff et al. (10). Isoe and coworkers (9) suggested that the biogenesis of β -damascenone starts with neoxanthin and its formation involved a Meyer-Schuster rearrangement of an allenic triol followed by a dehydration of 3-hydroxy- β -damascone to give β -damascenone (Figure 1).

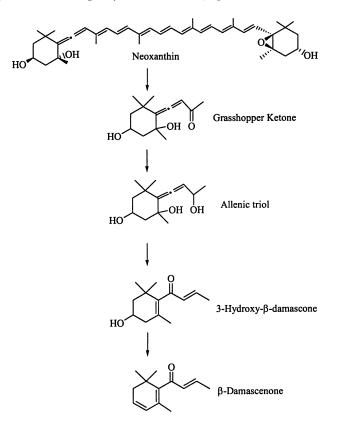


Figure 1. Proposed pathway of β -damascenone formation from neoxanthin (9).

All of the compounds shown in Figure 1 have since been observed in grape products (11-16). Isoe and coworkers (17) have also suggested that the biosynthesis of neoxanthin from zeaxanthin can occur in the presence of visible light and singlet oxygen. The feasibility of this mechanism was demonstrated *in vitro* by Tsukida et al. (18) who photo-oxygenated all-trans- β -carotene (sensitized by chlorophyll) and isolated an allenic carotenoid.

Ohloff et al. (10) proposed a similar biogenesis but focused on acetylenic alcohols as it was known from the work of Egger et al. (19,20) that the allenic carotenoid deepoxineoxanthin could be isomerized under acidic conditions to give the acetylenic carotenoid diatoxanthin (Figure 2).

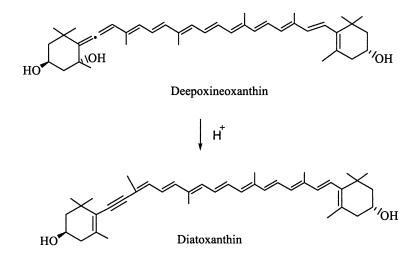


Figure 2. Isomerization of deepoxineoxanthin under acidic conditions.

Ohloff et al. (10) studied the acetylenic diol (Figure 3) which gave both 3hydroxy- β -damascone and β -damascenone in a ratio of 4:1 in 30 % sulfuric acid solution. Importantly, they showed that the transformation of 3-hydroxy- β damascone to β -damascenone proposed by Isoe et al. (9) did not occur under these strongly acidic conditions. Skouroumounis and Sefton (21) subsequently showed that the hydrolysis of the acetylenic diol at room temperature and at wine pH (pH 3.0) was very slow and after one year, only 1 % of β -damascenone and 21 % of 3-hydroxy- β -damascone was formed. Chemical transformation of this precursor is unlikely to account for the β -damascenone seen in young wines, but might yield small amounts of β -damascenone during bottle maturation.

Skouroumounis et al. (22) have shown that the allenic triol (which has the same oxidation state as the acetylenic diol) proposed by Isoe et al. (9) can give β -damascenone rapidly at wine pH and at room temperature, albeit in low yield. The two main products of the reaction were 3-hydroxy- β -damascone, and acetylenic diol in a ratio of 5.5:1 (cf. Figure 4).

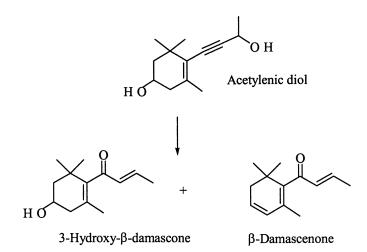
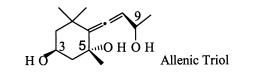


Figure 3. Acid-catalyzed formation of 3-hydroxy-β-damascone and βdamascenone (ratio 4 :1) from the acetylenic diol.





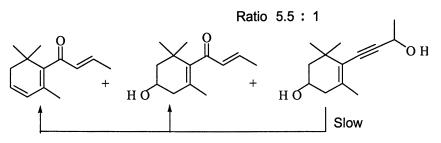


Figure 4. Acid-catalyzed degradation (pH 3, room temperature) of the allenic triol (for details cf. text).

The acetylenic diol (cf. Figure 4) could not have been an intermediate in the conversion of the allenic triol to β -damascenone and 3-hydroxy- β -damascone, because of its low reactivity at room temperature. The greater reactivity of α -allenic alcohols compared to α -acetylenic alcohols has been described by Olsson et al. (23). Two intermediates, tentatively assigned as megastigma-4,6,7-triene-diol (conjugated allenic diol) and the megastigman-3,5-dien-7-yn-9-ol (conjugated acetylenic alcohol) were observed in the hydrolysates of the allenic triol under mild conditions; these are discussed below (cf. Figure 10).

β-Damascenone found in at least some grape juices can therefore be accounted for by the hydrolysis of the allenic triol, as this β-damascenone is commonly accompanied by glycoconjugates of the other two products 3hydroxy-β-damascone and acetylenic diol in a ratio similar to that formed in the hydrolysis of the allenic triol (e.g. 13-15). The allenic triol has been recently identified in an enzymatic hydrolysate of a C18 reverse-phase isolate of a grape juice *cv*. Merlot (16).

Hydrolytic Studies on Glycoconjugates of Important Aroma Compounds

In 1984, Wilson et al. (24) reported an increase in monoterpene glycosides and a noticeable decrease in monoterpenes during the period of grape ripening and noted that the pool of glycoconjugates far outweighed the pool of free polyols found in juice/wine. Glycosylation of secondary metabolites occurs as the terminal step in many biosynthetic pathways (25). It has been shown that hydrolysis of crude extracts of glycosides from grapes can generate volatile fractions which influence wine flavor (26-28).

We have therefore studied the hydrolytic behavior of individual glycosides to see what role they play in flavor chemistry.

Hydrolysis of Geraniol and its β-D-Glucoside

The hydrolysis of geraniol (an allylic alcohol) and its β -D-glucopyranoside at 80°C, in 10% alcohol solution and at pH 3 was monitored (21). The two major products formed were linalool and α -terpineol (21) (Figure 5).

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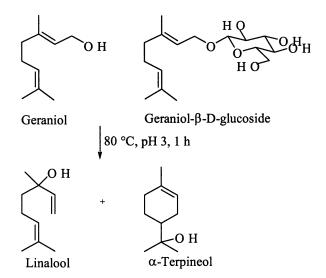


Figure 5. Acid-catalyzed conversion of geraniol and its β -D-glucoside.

After 1 hour, geraniol formed approximately ten times more linalool than did the corresponding β -D-glucopyranoside. Following longer reaction time linalool levels decreased as more α -terpineol was formed. At lower temperatures, i.e. 50°C, only three products linalool, α -terpineol and 2,6dimethyl-7-octene-2,6-diol were seen from the β -D-glucopyranoside whereas thirteen compounds were identified from the aglycon hydrolysis at this temperature (21,29). No significant amount of geraniol was formed from the glucoside. Ohta et al. (30) have reported similar reactivity for the β -Dglucopyranoside of geraniol under acidic conditions.

Hydrolysis of the Model Acetylenic Alcohol and its β-D-Glucoside

Interest was then focused on the reactivity of a model acetylenic alcohol and its β -D-glucopyranoside (Figure 6). Synthesis of this model propargyl alcohol and its glucopyranoside has been reported elsewhere (31).

At pH 3 and 100°C the reactivity of the acetylenic alcohol was low. After forty-six hours, approximately 26 % of the acetylenic alcohol had reacted to give two major products, the conjugated α , β -unsaturated ketone, formally

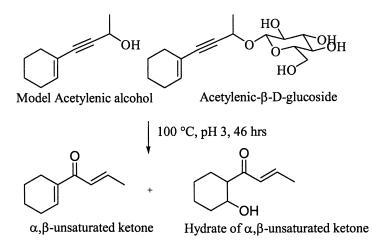
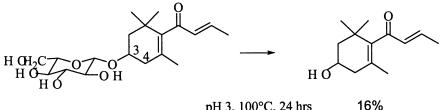


Figure 6. Acid-catalyzed conversion of the model acetylenic alcohol and its β -D-glucoside.

derived via a Meyer Schuster rearrangement (32,33) and a second product, tentatively identified from its mass spectrum as the hydrate of the α,β -unsaturated ketone (21).

The conversion of the acetylenic C9 glucoside to these products was found to be approximately three times slower than that of the aglycon. The aglycon was formed from the glucoside along with the unsaturated ketones.

These studies have shown that the two glucosides investigated are slower reacting than the corresponding aglycons but we were interested in seeing whether glycosylation of the alcohol group of a cyclohex-1-en-4-ol would enhance dehydration to give a C3-C4 double bond and in particular, whether the glucoside of 3-hydroxy- β -damascone would give β -damascenone. When the hydrolysis of the β -D-glucopyranoside of 3-hydroxy- β -damascone was carried out at pH 3.0 and 100°C for twenty-four hours, 16 % of the aglycon was obtained but no trace of β -damascenone could be detected (Figure 7). At pH 1.1 and 100°C, 3-hydroxy- β -damascone was obtained in 35 % yield after 8 hours. No elimination products were seen with the hydrolysis of the glucoside even under these forcing conditions.



pH 3, 100°C, 24 hrs 16%

pH 1.1,100°C, 8hrs 35%

Figure 7. Hydrolysis of the β -D-glucopyranoside of 3-hydroxy- β -damascone.

Implications for β-Damascenone Formation from the Allenic Triol

There are several pathways by which the allenic triol could be converted to its hydrolysis products. Loss of the C8 hydrogen and C5 hydroxyl group would lead to the acetylenic diol which under acidic conditions slowly gives both 3hydroxy- β -damascone and β -damascenone (Figure 8, R=H). By analogy with the model acetylenic alcohol (Figure 6) glycosylation of the acetylenic diol at C9 might be expected to slow down the ionization at this position thereby effectively allowing other transformations to occur within the molecule and increase the amount of β -damascenone formation. An example of glycoconjugation altering the formation of volatile wine components has been described (34), i.e. a higher proportion of vitispirane was formed when its precursor was glycosylated at the C3 position.

The synthesis of the acetylenic diol and its C9 β -D-glucoside which involved some thirteen steps has been reported previously (31). Consistent with the behavior of the glycosides described above, the acetylenic diol was found to be eight times more reactive than the C9 β -D-glucoside at 50°C and pH 3. After 28 days the major products were β -damascenone and 3-hydroxy- β -damascone, in a ratio of 1:18 from the acetylenic diol, and 1:11 from its glucoside (21). Thus at 50°C the proportion of β -damascenone generated was greater from the glucoside than from the acetylenic diol even though it was formed more slowly. It appears that by slowing the acid-catalyzed ionization of the propargyl alcohol function, the presence of the glycopyranosyl moiety promoted other competing transformations within the molecule as was predicted. The side chain of the C9 β -D-glucoside can either react directly to give Meyer-Schuster rearrangement products, or indirectly *via* the acetylenic diol (Figure 9).

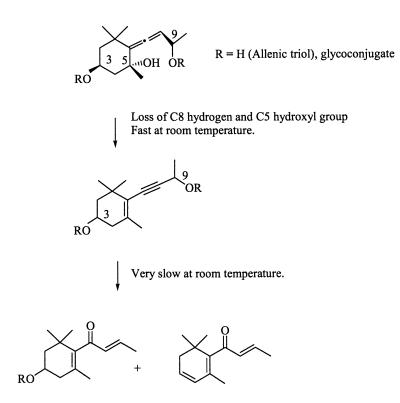


Figure 8. Conversion of the allenic triol and its β -D-glucoside.

The ratio of β -damascenone : 3-hydroxy- β -damascone formed by the hydrolysis of acetylenic diol or its β -D-glucoside is also dependent on the reaction temperature (21,35). The ratio is a reflection of the selectivity of ionization exhibited at the different temperatures, with the proportion of β -damascenone in the products from both the aglycon and the glycoside being greater at a higher temperature. When fruits undergo high temperature processing this can effectively increase the amount of β -damascenone, if the acetylenic-C9- β -D-glucoside or aglycon, is present as a precursor. Furthermore, heating wine/juice is not only likely to accelerate the generation of β -damascenone but could ultimately lead to a higher yield of this compound than could be obtained by natural wine aging. The acetylenic-C9- β -D-glucoside has

been isolated as a natural product from Riesling wine while the aglycon has been found in grape juices and wine (13-15, 35-37).

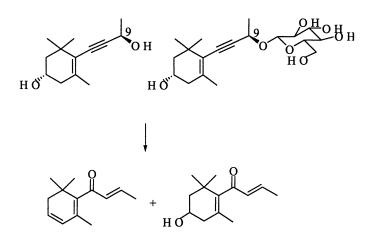


Figure 9. Acid-catalyzed conversion of the acetylenic diol and its β -Dglucoside.

Two intermediates, observed in the hydrolysates of the allenic triol under mild conditions and tentatively identified were megastigma-4,6,7-triene-3,9-diol (conjugated allenic diol, I) and megastigman-3,5-dien-7-yn-9-ol (conjugated acetylenic alcohol, II) (cf. Figure 10, R=H). The proposed bifunctional intermediate megastigma-4,6,7-triene-3,9-diol I can be formed from the elimination of the C4 hydrogen and C5 hydroxyl function under acid conditions. If the C3 hydroxyl group of the diol is glycosylated, then this should favor ionization at the C9 position and the formation of the corresponding glycosides of 3-hydroxy- β -damascone. If on the other hand the C9 position was glycosylated, then this should diminish ionization at the C9 and thereby favor ionization at C3. On this basis, we might expect C9-glycoconjugates of the allenic triol to be the most important precursors of β -damascenone.

A C9- β -D-glucopyranoside (as the polyacetate) of the allenic triol has been isolated from *Lycium halimifolium* Mill. (38) at the natural plant pH of 4.0 and an undetermined disaccharide has been found in apples *Malus sylvestris* Mill., cv. Jonathan (39). The allenic triol has also been identified in a glycosidase enzyme hydrolysate of a crude glycosidic fraction from Merlot grape juice (16).

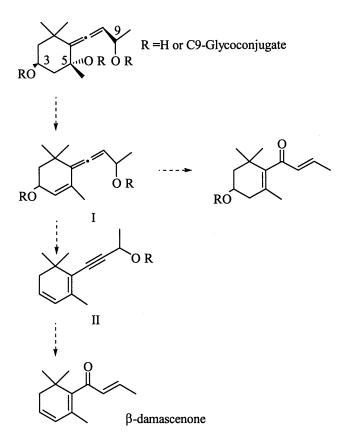


Figure 10. Structures of tentatively identified intermediates I and II.

Finally, the early work by the Swiss flavor and fragrance company *Firmenich SA* on β -damascenone formation is of particular relevance to the sequence shown in Figure 10. Two precursors synthesized by the company and patented involved C9 protected side chains (40,41). In both cases, β -damascenone was obtained as the predominant product (Figure 11). The tertiary butyl ether is simply a derivatised form of the conjugated allenic diol, while the tetrahydropyranyl ether is an acetal of the hydrate of the conjugated acetylenic alcohol (Figure 11). In both cases, the yield of β -damascenone that might have arisen from the unprotected diols is not reported. It is possible that these protecting groups may have slowed down the rapid ionization of the C9-hydroxyl group thereby allowing other transformations to occur within the molecule, leading to favorable β -damascenone formation.

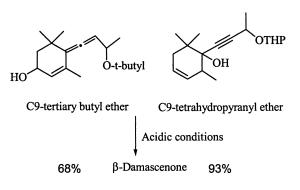


Figure 11. Synthetic precursors to β -damascenone (40,41).

Conclusions

Most of the intermediates and by-products proposed in the hypothetical genesis of β -damascenone from neoxanthin in grapes and wine have now been identified, directly or indirectly, in grape products. These compounds occur, for the most part, as glycoconjugates. Further work will confirm the degree to which glycoconjugation might steer the catabolism of neoxanthin towards or away from β -damascenone.

Acknowledgements

We thank Professor P. Winterhalter for his advice and encouragement and the Grape and Wine Research and Development Corporation for funding this research.

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Chapter 18

C₁₃-Norisoprenoid Aglycon Composition of Leaves and Grape Berries from Muscat of Alexandria and Shiraz Cultivars

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 C_{13} -norisoprenoid glycoconjugates from Muscat of Alexandria and Shiraz leaves and grape berries isolated through adsorption on Amberlite XAD-2 resin were subjected to enzymatic hydrolysis and the released aglycons were analyzed by GC-MS. C_{13} -norisoprenoids were more abundant in the leaves than in the berries. Differences were observed with regard to the C_{13} -norisoprenoid pattern between leaves and berries. 3-Hydroxy- β -damascone, a major aglycon of grape berry C_{13} -norisoprenoid glycosides, was not detected in the leaves. Compositional differences were also observed between Muscat and Shiraz leaves. 3-Oxo- α -ionol, and its retro derivatives were more abundant in Muscat than in Shiraz leaves. Conversely, Shiraz leaves were richer in 7,8dihydroionone derivatives, such as megastigmane-3,9-diol and 3-oxo-7,8-dihydro- α -ionol.

The contribution of glycoconjugates to wine flavor through the release of volatiles is now well established (1-3). Potent odorants or their precursors, mainly monoterpenes, C₁₃-norisoprenoids and shikimic compounds, accumulate

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in grape berries as flavorless glycoconjugates. After hydrolysis the latter constituents can yield odor-active aglycons or aglycons which are themselves precursors of flavor compounds as it has been observed for most of the C_{13} norisoprenoid aglycons (1,3,4).

The qualitative as well as quantitative C_{13} -norisoprenoid composition of berries from several wine cultivars has been studied hitherto (3,5,6). In the case of grapevine leaves, however, our knowledge is quite limited. Except for the Riesling variety, little information is available so far (7). The aim of the present work was to study the C_{13} -norisoprenoid aglycon composition of grape berries and vine leaves from two cultivars, i.e. Muscat, a monoterpene-dependant variety, and Shiraz, a variety largely used in winemaking in order to assess the norisoprenoid flavor potential of vine plant and to have some insight into biochemical pathways involved. The data given here are related to the aglycon analysis of glycosidic extracts obtained from the samples by XAD-2 extraction and enzymatic hydrolysis. It should be noticed that most compounds mentioned here, particularly norisoprenoids, are mainly present in glycoconjugated forms in grape berries and leaves. This occurrence was confirmed in the present study.

Analysis of Glycosidically Bound Compounds

For the isolation of glycoconjugates the juice obtained by crushing grape berries and the water soluble fraction of methanolic extracts of vine leaves were loaded on Amberlite XAD-2 resin (8). The glycosidic extract was treated with a mixture of fungal-derived enzyme preparation possessing the relevant glycosidase activities to liberate the aglycons. The enzymatically released aglycones were then extracted and analyzed by GC/MS. The amounts of aglycons were determined as equivalent of 4-nonanol used as standard. Analyses were performed in triplicate.

Aglycon Composition of Muscat and Shiraz Leaves and Berries

About 90 volatiles were identified in enzymic hydrolysates of glycosidic extracts, thirty-four being C_{13} -norisoprenoids. Thirty of these compounds were detected in leaves and seventeen in berries. The total amounts of volatiles released by enzymatic hydrolysis from the glycosidic extracts of Shiraz and Muscat leaves were 356 and 433 mg/kg respectively. They were much higher than the total levels found in Shiraz and Muscat berries which were 4.7 and 6.9 mg/kg respectively, i.e. about 1.5% of that found in the leaves. About half the total level of leaf volatiles were C_{13} -norisoprenoids (cf. Figure 1). The other

compounds belonged to the classes of shikimates, monoterpenes, six-carbon alcohols, and other aliphatic alcohols (in decreasing order of abundance). Monoterpenes were twice as abundant in Muscat than in Shiraz leaves, which correlates with the abundance of these compounds in Muscat berries (2,9). With regard to the distribution of aglycons in berries, monoterpenes dominated in the Muscat cultivar (2,9), followed by shikimate derivatives and C_{13} -noriso-prenoids. The latter two classes of compounds were most abundant in Shiraz berries.

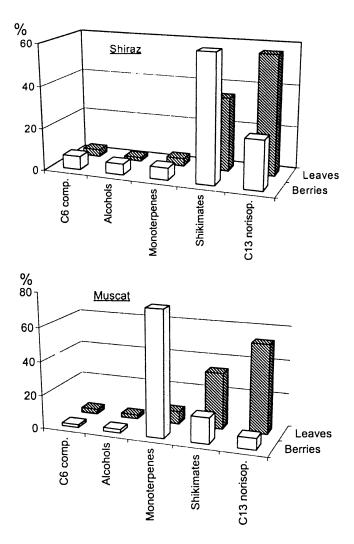


Figure 1. Distribution (%) of glycosidically bound compounds in leaves and berries of Shiraz and Muscat cultivars.

In Carotenoid-Derived Aroma Compounds; Winterhalter, P., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2001. Most importantly, high levels of C_{13} -aglycons were detected in leaves (200 and 250 mg/kg for Shiraz and Muscat, respectively), while relatively low amounts were detected in the berries (i.e. 1.07 mg/kg for Shiraz and 0.47 mg/kg for Muscat). This amounts to 0.5% of that found in the leaves. Similar results were reported for Riesling leaves (7). These high levels of norisoprenoids could be attributed to the abundance of carotenoids in the leaves compared to the low carotenoid concentration in the berries. For example, the sum of β -carotene, lutein, violaxanthin and neoxanthin concentration in mature Muscat berries was found to be approximately 2 mg/kg versus 400 mg/kg in the leaves.

Only quantitative differences were observed between the C₁₃-norisoprenoid berry compositions from Shiraz and Muscat cultivars. The major aglycons were 3-hydroxy- β -damascone, 3-oxo- α -ionol and vomifoliol in accordance with data reported from other cultivars (5,6). However the pattern of C₁₃-norisoprenoid aglycons in leaves was found to be different than that found in berries. Most major compounds identified in leaves were only minor compounds in the berries and vice versa. Furthermore 3-hydroxy-ß-damascone could not be detected in Shiraz and Muscat leaves. A similar observation was reported for Riesling leaves (7). This is surprising because we have detected in vine leaves its' plausible carotenoid progenitor which is neoxanthin. The absence of 3-hydroxy-B-damas-cone in leaves can be explained by the presence of only trace amounts of one of its known precursor which is 3-hydroxy-7,8-dehydro-ß-ionol. 3-Hydroxy-7,8-dehydro-ß-ionol also acts as precursor of ß-damascenone, the well-known potent flavor compound (3,10). Interestingly, β -damascenone levels in berries of both cultivar studied were in the range of those of other norisoprenoids. This suggests the existence of different pathways in the metabolism of neoxanthin derivatives in leaves and berries.

Compositional differences were clearly observed between Muscat and Shiraz leaves. 3-Oxo- and 3-hydroxy-7,8-dihydro-ionone derivatives 1,4,5 were much more abundant in Shiraz leaves compared to Muscat leaves (Figure 2). (Note that compounds 1 and 2 were only tentatively identified). Compound 4, a fully saturated megastigmane-3,9-diol, was identified in our laboratory for the first time in vine leaves (11). It was one of the major norisoprenoidic aglycons (33.9 mg/kg) in Shiraz leaves. Neither Shiraz berries nor Muscat leaves contained this compound which might be converted by dehydration to less polar C_{13} -derivatives, i.e. potential odorants. The absolute stereochemistry of 4 was recently determined (12). Diol 4 occurs mainly in monoglucosidic form either glucosylated at C-9 or at C-3, e.g. in the leaves of Alangium premnifolium (13). Disaccharidic conjugates of 4 were also tentatively identified in lower amounts by GC/MS in form of their trifluoroacetylated derivatives. These results as well as those reported for Riesling leaves (7) support the hypothesis of the dominance of monoglucosides in vine leaves, in contrast to grape berries where the ratio monoglucosides/diglycosides is much more balanced.

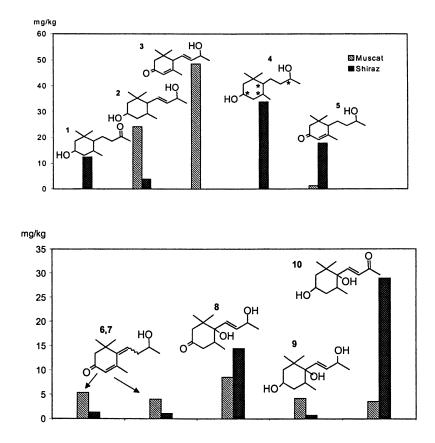


Figure 2. Major differences in C_{13} -norisoprenoid composition of Muscat and Shiraz leaves.

The abundance of ionone derivatives with a saturated 7,8-double bond in Shiraz leaves compared to Muscat leaves suggested a high reductase activity in the former variety. Indeed, 3-oxo- α -ionol (3) which is one of the major aglycons in Muscat leaves (48 mg/kg) was not detected in Shiraz leaves in which more saturated derivatives of this compound occurred. Furthermore Muscat leaves contained higher levels of the retro isomers 6 and 7 of 3-oxo- α ionol compared to Shiraz leaves. The differences were less important between Muscat and Shiraz leaves for other ionone derivatives, these are 3-hydroxy-7,8dihydro- β -ionone, 3-hydroxy-7,8-dihydro- β -ionol, and 3-hydroxy- β -ionone.

As in Riesling leaves (7), grasshopper ketone, an oxidized precursor of β damascenone, was detected in Muscat leaves among the major norisoprenoid aglycons. However this allenic ketone was hardly detectable in Shiraz leaves. This could be attributed to its reduction to other tri-oxygenated C₁₃-norisoprenoids which might not be amenable to GC analysis as reported for its corresponding alcohol (14).

Among tri-oxygenated C_{13} -compounds, the level of 3,6-dihydroxymegastigm-7-en-9-one (10) was much higher in Shiraz leaves than in Muscat leaves. This compound together with compound 9 was also detected in starfruit (15). Contrary to the di-oxygenated derivatives, the side chain double bound of the major tri-oxygenated C_{13} -compounds in Shiraz leaves was not saturated.

Conclusions

In conclusion, this work highlights the occurrence of high levels of C_{13} norisoprenoidic aglycons in vine leaves compared to grape berries. This study also shows that vine leaves could be a good model for the search of various enzymes (carotene-oxygenases, reductases, hydroxylases, glycosyltransferases etc.) involved in the biogenesis of norisoprenoids due to the abundance and diversity of related compounds. In view of the analytical data given here, biosynthesis of norisoprenoids in grape berries seems to be independent from norisoprenoid formation in the leaves.

For future studies the following questions remain: (i) Determination of the structure of some minor norisoprenoids that have been detected in the leaves together with the characterization of the structures of sugars involved, and (ii) evaluation of the sensory potential of norisoprenoidic aglycons and derived odorants.

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Chapter 19

Effect of Sunlight Exposure on Norisoprenoid Formation in White Riesling Grapes

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Norisoprenoids are volatile secondary metabolites which are important aroma constituents of a number of grape and wine varieties. The norisoprenoids are thought to arise from carotenoid breakdown and occur in grapes as glycosidically However, the factors which influence bound precursors. carotenoid breakdown and norisoprenoid formation in grapes are not well understood. In this study we evaluated the effect of seven levels of sunlight exposure (4%, 18%, 31%, 37%, 50%, 71%, and 97% expressed as percent of full sun exposure) on norisoprenoid concentrations in White Riesling. Our results suggest that viticultural practices which affect fruit exposure to sunlight may influence norisoprenoid composition In White Riesling, TDN (1,1,6of grapes and wines. trimethyl-1,2-dihydronaphthalene) Riesling acetal and (2,2,6,8-tetramethyl-7,11-dioxatricycloundec-4-ene) levels increased at sunlight exposures above 20% of full sun exposure. Changes in grape berry temperature with increasing sunlight exposure may also have an influence on norisoprenoid levels.

In Carotenoid-Derived Aroma Compounds; Winterhalter, P., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Norisoprenoids contribute characteristic aromas to many varieties of Vitis vinifera. For example, in Chardonnay, Williams and co-workers (1 - 3) showed that "grassy", "tea", "lime", "honey", and "pineapple" aromas were derived from norisoprenoids and their precursors. Both red and white non-floral varieties, including Chenin blanc, Semillon, Sauvignon blanc, Cabernet Sauvignon, and Syrah, are known to contain significant levels of norisoprenoids (4 - 10). Even in the floral varieties (e.g., White Riesling and Muscat) which derive most of their aroma impact from terpenes, norisoprenoid concentrations up to 40% higher than those of terpenes have been observed (11).

Norisoprenoids in grapes are thought to arise from photochemical and enzymatic degradation of carotenoid precursors found in the skin and pulp. The major carotenoids are β -carotene and lutein present in levels of 1-2 ppm (12-14). Lower levels (ppb) of several other oxygenated carotenoids (i.e., xanthophylls), including neoxanthin, flavoxanthin, 5,6-epoxylutein, luteoxanthin, and violaxanthin have also been identified in grapes (12, 14). The chemical and biochemical processes involved in carotenoid degradation and subsequent norisoprenoid formation are not well understood and are further discussed in other chapters of this proceedings.

Many norisoprenoids occur in grapes as glycosidic precursors. Enzymatic and acid cleavage during crushing, fermentation and bottle-aging result in cleavage of the bound sugar moiety releasing the free norisoprenoid aglycone. These reactions may be particularly important for the bottle-aging flavor of many wines, including White Riesling.

Viticultural Factors Which May Influence Norisoprenoid Levels in Grapes

Climate

Cooler regions generally produce grapes with lower carotenoid levels (13). Similarly, Marais and co-workers (7, 8) observed that levels of two norisoprenoids, TDN and *trans*-vitispirane, were lower in White Riesling wines from relatively cool regions such as Northern Italy and Germany compared to wines from warmer South African regions (Regions I and II vs Regions III, IV, and V, respectively; classified according to Winkler et al. (15)). However, only a limited number of norisoprenoids were identified by Marais et al. and further studies on the effects of climatic variation on a larger number of norisoprenoids and their carotenoid precursors need to be done.

In the studies by Marais and co-workers (7, 8), sunlight exposure differed substantially between the cool and warm regions, with average daily sunshine hours being lower in the cooler European regions compared to the South African regions (4-6 vs 9-11 sunshine hours per day, respectively). Microclimates within vineyard sites and within the grapevine canopy can also be quite variable making it difficult to directly evaluate climatic differences between regions. Other variables including clonal variation and differences in rainfall and crop yield may influence production of secondary metabolites, including carotenoids and norisoprenoids. These factors highlight just a few of the difficulties encountered when attempting to study viticultural variables which may influence grape and wine quality.

Sunlight

Marais et al. (6) observed that levels of several norisoprenoids (TDN, hydroxy-TDN, ionols, actinidols, grasshopper ketone, vitispirane. and vomifoliol) were significantly higher in sun-exposed White Riesling and Chenin blanc grapes compared to shaded grapes of the same varieties. In this study, sun-exposed grapes received direct sunlight in the morning on the southern side of the rows and in the afternoon on the northern side of the rows. Shaded grapes were shielded by the leaves from direct sunlight exposure. Actual differences in photosynthetically active radiation (PAR) between the two treatments were not measured. Interestingly, β-damascenone levels did not appear to be influenced by sunlight levels.

Razungles et al. (14) observed that the timing of sun-light exposure influenced both carotenoid biosynthesis and norisoprenoid levels in Syrah grapes. Sunlight exposure before veraison resulted in increased carotenoid levels, consistent with previous studies showing that carotenoid biosynthesis is a light-induced process and that synthesis stops at veraison. Interestingly, the ratio of epoxyxanthophylls to non-epoxyxanthophylls appeared to be influenced by sunlight exposure. Similar to results observed by Marais et al. (6), Razungles et al. found that sunlight exposure after veraison increased norisoprenoid levels in the Syrah grape berries. Light intensity of the sunexposed berries was about 10 times that of the shaded berries, however, differences in temperatures of up to 5° C were also observed between the two treatments. Therefore temperature effects should not be completely excluded as at least one partial explanation for the observed results.

Grape Maturity

Norisoprenoid levels typically increase during grape ripening. The increase in norisoprenoid levels which starts at veraison is accompanied by a steady decrease in total carotenoid concentration (12, 16). Strauss et al. (17) showed a linear, positive relationship between levels of three norisoprenoids (Vitispirane, TDN and β -damascenone) and sugar accumulation in White Riesling. The results suggest that picking at earlier maturity may be one way to control TDN formation and development of bottle-aging character in Riesling wines from warm regions.

Objectives

The above studies have highlighted the need for an in depth understanding of the effects of viticultural variables on the production of secondary metabolites, including norisoprenoids in grapes. While it is clear from the work of Marais et al. (6) and Razungles et al. (14) that sunlight can influence norisoprenoid levels in both red and white varieties, the light intensity required to elicit these effects is not clear. For example, it is not known if norisoprenoid levels are enhanced with increasing sunlight levels or if there is an upper limit of sunlight exposure beyond which no further increase in norisoprenoid concentrations is observed. Therefore, the current study was undertaken to further evaluate the effects of a range of sun exposure levels on the concentrations of free and glycosidically bound C_{13} -norisoprenoids in White Riesling grapes.

Materials and Methods

Grapes

The study took place during the 1999 growing season using own rooted White Riesling vines located at the UC Davis Vineyard, Davis, CA. The vines were 25 years old, planted in a north-south row orientation with 20' x 10' vine spacing on an expanded lyre trellising system.

Shade Treatments

Shade cloth of differing densities (John Mahaney Co., Sacramento, CA) was used to provide variable levels of sunlight exposure to the grape clusters. A 60 cm x 60 cm piece of cloth was stretched across a wire frame which was placed in the ground so that the cloth was approximately 15 cm from the clusters. Spacing between adjacent screens was ~ 25 cm and air was allowed to move freely between the frame/shade cloth and the grape vine/clusters. Leaves were removed and shade cloth applied to give the following levels of sunlight to the cluster (expressed as percent of ambient light): 4%, 18%, 31%, 37%, 50%, 71%, and 97%. In addition, a natural shade treatment (which corresponded to \sim 3% of full sun exposure) was obtained by maintaining the original leaf shading of the clusters. Treatments were established at veraison and actual photosynthetically active radiation (PAR) at each cluster was measured on several days and at several times throughout the day (9:00, 12:00, and 15:00 Pacific Daylight Time) to ensure that shade levels remained consistent throughout the experiment. PAR was measured using a handheld Li-Cor LI-189 quantum sensor (Li-Cor, Inc., Lincoln, NE).

Each cordon of a vine was divided into two or three sections which each yielded two or three clusters. To each section, a randomly assigned shading treatment was applied, and each treatment was replicated two times on separate vines.

Temperature Measurements

Berry temperature was measured at the same time as PAR using a handheld Omega HH 23 Temperature Monitor with dual hypodermic thermocouples (Omega Engineering, Inc., Stamford, CT). The probe was inserted into the berry center and a shielded probe, next to the berry, monitored ambient air temperature.

Harvesting

All clusters were harvested at 20-23°Brix, determined in the vineyard with a handheld refractometer. Clusters were picked, cooled to $\sim 10^{\circ}$ C, transported to the laboratory, and stored frozen at -20°C prior to extraction and analysis.

Isolation and Analysis of C₁₃ Norisoprenoids

Grapes from each of the treatments were crushed and gently pressed through a cheese cloth to obtain the juice. Free volatiles were isolated from ~ 150 mL juice by liquid/liquid extraction using pentane/diethyl ether (1:1 v:v) as the extracting solvent. Internal standard (2-octanol) was added to the juice prior to the extraction. After the extraction was complete (18 hours) the organic phase was dried over anhydrous sodium sulfate and concentrated to 1.5 mL on a Vigreux column. The concentrated extract was stored at < 10°C until GC analysis. Immediately prior to GC analysis, the extract was further concentrated to 100 μ L under a gentle nitrogen stream.

Using the juice remaining from the liquid/liquid extraction, bound volatiles were isolated using simultaneous distillation and extraction (SDE). Any pentane/ether remaining in the juice was first removed by distillation under vacuum at room temperature (Rotovap). A citrate/phosphate buffer (pH 3.2) was added to the juice and 1-heptanol added as an internal standard. The solution was extracted with pentane/diethyl ether (1:1 v:v) by SDE for 1 hour. The extract was dried over sodium sulfate and concentrated to 1.5 mL on a Vigreux column and the concentrated extract stored at < 10°C until GC analysis. Immediately prior to GC analysis, the extract was further concentrated to 100 μ L under a gentle nitrogen stream.

All extracts were analyzed by gas chromatography with mass spectrometric detection (GC-MS) using a Hewlett Packard 6890 GC with a 5972 MSD and ChemStation Software. Helium was the carrier gas at a flow rate of 0.8 mL/min and the MSD interface temperature was 260°C. A 30 m x 0.25 mm i.d. x 0.25 μ m film thickness DB-Wax (polyethylene glycol) column (J&W Scientific, Folsom, CA) was used for all analyses. The oven temperature was programmed from an initial temperature of 50°C to a final temperature of 240°C at a rate of 4°C/min. The oven was held at the final temperature (240°C) for 25 min. The injector temperature was 230°C and 1 μ L of concentrated extract was injected in the splitless mode.

Peak identity of individual peaks was confirmed by comparison of retention times and mass spectra (obtained in full scan mode) with those of authentic standards injected under identical condition.

For quantitation, the GC-MSD was operated in Selected Ion Monitoring (SIM) mode. Peak areas for m/z 172 and 207 were used for quantitation of TDN and Riesling Acetal, respectively. These masses correspond to the molecular ion for TDN and M-1 for Riesling acetal. Concentration of each peak was calculated relative to the peak area of the internal standard, 2-octanol or 1-heptanol. Norisoprenoid levels were calculated on a per berry basis as well as

on a concentration basis. No significant difference in results was observed between these two methods and so all results are reported on a concentration basis.

Results and Discussion

The norisoprenoids TDN and Riesling acetal were positively identified in the extracts from the White Riesling grapes. Further work is underway to accurately identify and quantify other norisoprenoids which are present in the grapes at trace levels.

Levels of bound TDN and Riesling acetal in the juice were generally higher than free levels at all sunlight exposures (Figures 1 and 2). These results are consistent with literature reports indicating that norisoprenoids are generally present as glycoconjugates or polyol precursors which are easily degraded under acidic conditions (4, 18 - 20).

Levels of bound TDN and Riesling acetal appeared to increase at each successive level of sunlight exposure above ~ 20% of full sun exposure (Figure 1). Free norisoprenoid levels also increased at higher sun exposures, but the effects were not as pronounced. Temperature in the berry ranged from 31° C at the lowest sunlight levels to 33° C at the highest sunlight levels. While the observed difference in temperature is not extreme, temperature cannot be excluded as a factor which may influence norisoprenoid levels in grapes. Winterhalter (21) has proposed that Riesling acetal and TDN can arise from the same hemiacetal precursor (Figure 2). Further work is needed to determine if the concentration of this precursor is also enhanced with increasing sunlight exposures.

Preliminary results indicate that TDN levels were significantly higher in berries from the naturally shaded vines compared to grapes from vines which had the applied shade cloth treatment with a similar PAR level (Figure 3). Reasons for this observed result are not known, however, Schultz et al. (22) have shown that carotenoid concentrations in grape berry skins can be influenced by exposure to UV-B irradiation. The applied shade cloth used in the current study filtered light between 300 and 700 nm while leaves also filter and reflect light of other wavelengths which may influence carotenoid synthesis and degradation. Further work is needed to confirm the results observed in this study and to determine the effect of exposure to light in the ultraviolet and infrared regions.

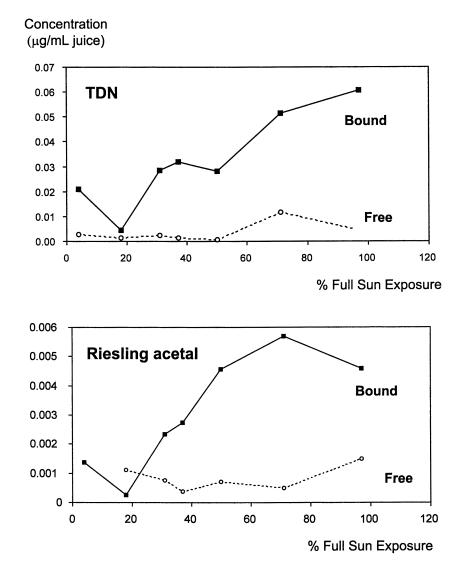


Figure 1. Bound and free levels of TDN and Riesling acetal in White Riesling grape juice at increasing levels of sunlight exposure (n = 2).

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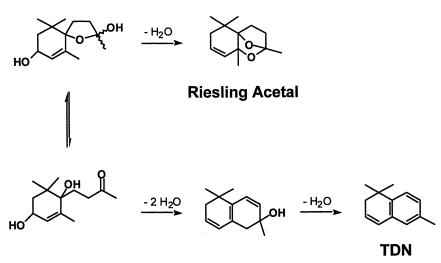


Figure 2. Proposed formation of Riesling acetal and TDN from hemiacetal precursor (2,6,10,10-tetramethyl-1-oxaspiro[4.5]dec-6-ene-2,8-diol) in Riesling wines.

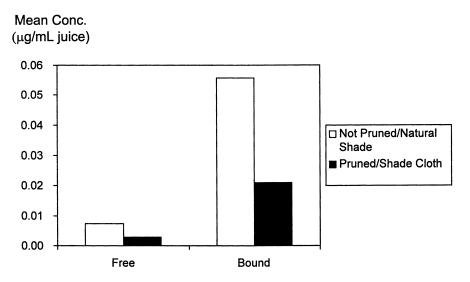


Figure 3. Effect of leaf removal on TDN levels in White Riesling berries. Photosynthetically active radiation (PAR) was 3-4% of ambient, obtained either from natural leaf shading or from application of shade cloth (n = 2).

In Carotenoid-Derived Aroma Compounds; Winterhalter, P., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2001. Temperature and light exposure affect many important physiological processes in grape berries including sugar accumulation, breakdown of acids, and anthocyanin synthesis. However, little is known about the effects of sunlight on flavor formation in grapes. In the current study we showed that sunlight exposure greater than ~20% of full sun exposure beginning at veraison increased bound TDN and Riesling acetal levels in White Riesling grapes. In addition, leaf removal at veraison decreased TDN levels in the berries. Further work is needed to confirm these results in other grape varieties and to determine possible explanations for the observed effects. Knowledge of the factors which influence synthesis and degradation of secondary metabolites, including carotenoids and norisoprenoid flavor compounds, will be essential in order to allow viticulturists and winemakers to optimize the levels of the aroma relevant compounds in grapes and wines.

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Chapter 20

The Significance of 1,1,6-Trimethyl-1,2-Dihydronaphthalene in the Production of High Quality Riesling Wines

Johann Marais

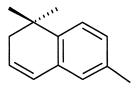
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1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN) is considered an important impact aroma component of aged Riesling wines. This component occurs in grapes as non-volatile precursors and is liberated by acid-catalyzed hydrolysis in wine during aging. It is the main contributor to the kerosenelike character, which may become detrimental to wine quality when present at too high intensities. The purpose of a series of studies was to gain knowledge about the factors that affect the formation of TDN and to apply this knowledge in restricting the occurrence of TDN in wine to sensorially acceptable levels. It was demonstrated that thermal degradation of lutein, one of the main carotenoids of Riesling grapes, yielded, amongst others, TDN. Canonical and stepwise discriminant analysis on the concentrations of various aroma components permitted the prediction of the origin of Riesling wines with a high degree of accuracy. One of the selected components with a highly significant discriminatory power was TDN, which was found to be higher in concentration in wines produced in warm than in cool climate countries. During grape ripening, concentrations of precursors of TDN, as well as other C₁₃-norisoprenoids, increased and were significantly in sun-exposed shaded higher compared to grapes.

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Furthermore, the concentration of TDN increased during aging of Riesling wine. The higher the temperature or the longer the storage time, the higher the TDN concentration and the stronger the kerosene-like character.

Numerous studies have demonstrated the importance of impact aroma components to the character and quality of grapes and wines (1-4). When key components responsible for specific aromas are known and quantified, they can be utilized as a tool to optimize viticultural and oenological practices to obtain maximum grape and wine quality. As far as C_{13} -norisoprenoids are concerned, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) is of special interest. During aging, Riesling wines may develop a strong kerosene-like character, which is mainly caused by TDN. This component occurs in grapes as non-volatile precursors (5-8) and is liberated by acid-catalyzed hydrolysis in wine during aging. It has a threshold value of 20 ppb in wine (4). When present at too high intensities, the kerosene-like aroma becomes a negative quality characteristic of Riesling wine, a phenomenon often observed in warm climate wine-producing countries.



TDN

This paper summarizes research results on the effects of carotenoid precursors, macroclimate, microclimate, grape maturity and wine aging on TDN levels in Riesling wines. The purpose was to gain knowledge about the subject and to apply it in restricting the occurrence of TDN in wine to sensorially acceptable levels.

Results and Discussion

Carotenoid Precursors

The possibility exists that TDN may derive from carotenoids via glycosidically-bound precursors, or other yet unidentified pathways. To determine whether the major carotenoids in grapes, i.e. lutein and β -carotene, may yield TDN, pure samples thereof were heated in sulphuric acid and ethanol/tartaric acid media, adjusted to pH 1 and 3, respectively (9). When the main degradation products of these two carotenoids are viewed, it is clear that ßcarotene yielded no TDN, while it was a major product of lutein (Table I). Lutein may therefore be an original precursor of TDN under these conditions. Carotenoid concentrations decreased during grape ripening (10) and were found to be higher in concentration in grapes cultivated in warm regions compared to those from cool regions (11). Based on the fact that carotenoids are the precursors of certain norisoprenoids, higher levels of the latter may be anticipated in grapes and wines This was indeed the case with TDN, as well as transfrom warm regions. vitispirane, which were present at higher concentrations in South African bottleaged Riesling wines than in German and Italian wines of similar ages (12) (see also next section).

Macroclimate

To determine the effect of macroclimate/region on TDN development, commercial Riesling wines from different vintages and regions in South Africa, Germany and Italy were analyzed for free TDN, as well as other C_{13} -norisoprenoids and monoterpenes (12). Compared to the cooler European regions, the Riesling wines from the warmer South African regions contained on average 78 % higher TDN concentrations. Canonical and stepwise discriminant analysis on the concentrations of some monoterpenes and C_{13} -norisoprenoids, permitted the prediction of origin with a high degree of accuracy (Figure 1). The fact that TDN (Table II) was selected as one of the components with a highly significant discriminatory power is in support of claims that TDN, with its pronounced kerosene-like aroma, is in fact responsible for prominent aged bouquet differences between Riesling wines from warm versus cool regions.

	Media			
Degradation Product	$H_2SO_4^{-1}$	EtOH/TA ²		
β-Carotene				
1) 6-Methyl-2-heptanone	x	-		
2) 6-Methyl-5-hepten-2-one	x	-		
3) β-Cyclocitral	х	х		
4) β-Ionone	х	х		
5) 5,6-Epoxy-\u00d3-ionone	х	х		
6) Dihydroactinidiolide	x	х		
7) 4-Oxo-β-ionone	х	х		
Lutein				
1) 1,1,6-Trimethyl-1,2-dihydronaphthalene	x	-		
2) 2,3-Dehydro-α-ionone (2 isomers)	x	х		
3) 3,4-Dehydro-β-ionone	x	-		
4) 3-Oxo-α-ionone	x	x		
5) 3-Hydroxy-β-ionone	x	x		

Table I. Major Thermal Degradation Products of B-Carotene and Lutein

¹ β -Carotene and lutein heated under nitrogen at 50°C for 4 h in a H₂SO₄ solution, adjusted to pH 1 with distilled water. ² β -Carotene and lutein heated under oxygen at 60°C for 3 hours in a 10 % aqueous ethanol solution, adjusted to pH 3 with tartaric acid.

SOURCE: Adapted with permission from reference 9. Copyright 1992.

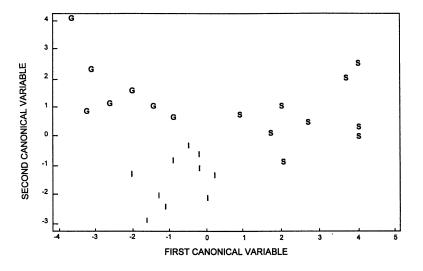


Figure 1. A canonical plot showing discrimination between Rieslings from South Africa (mainly regions III) (S), Germany (regions I) (G) and Northern Italy (mainly regions II) (I) on the basis of six free monoterpenes and C₁₃norisoprenoids. Regions classified according to reference 16. (Reproduced with permission from reference 12. Copyright 1992.)

Table II. Discriminant Variables Selected in Order of Importance fo	r the
Classification of Riesling Wines from South Africa, Germany and I	taly

Free Compound	F-value to enter	Bound Compound	F-value to enter
trans-Vitispirane 1,1,6-Trimethyl-1,2- dihydronaphthalene alpha-Terpineol trans-1,8-Terpin trans-Furan linalool oxide Diendiol-1	12.729** 8.967** 7.878** 5.678* 3.779* 2.258*	2-Hydroxy-1,8-cineole cis-Furan linalool oxide cis-Pyran linalool oxide Megastigma-5,8-dien- 3,7-dione cis-8-Hydroxylinalool Diendiol-1	16.319** 5.946* 5.688* 4.535* 4.254* 3.979*

**Highly significant ($p \le 0.01$). *Significant ($p \le 0.05$).

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Canopy Microclimate and Grape Maturity

The effect of sunlight, shade and degree of ripeness on potentially volatile C_{13} -norisoprenoid concentrations in Riesling grapes and wines was investigated (13). Grapes were sampled over three weeks between approximately 16°Brix (véraison) and 21°Brix (ripeness). Norisoprenoids were released from their glycosidically-bound forms by acid and enzymatic hydrolysis.

The concentration of released TDN was significantly higher in grapes exposed to sunlight than in shaded grapes, a tendency which occurred at all sampling stages (Figure 2). A similar tendency occurred in the wines made from grapes harvested at the third and fourth sampling stages. Furthermore, a significant increase in the acid-released TDN concentration was observed in the grapes with an increase in ripeness. Again, this finding was reflected in the corresponding wines. With few exceptions, other norisoprenoid concentrations showed tendencies similar to that of TDN (Tables III and IV).

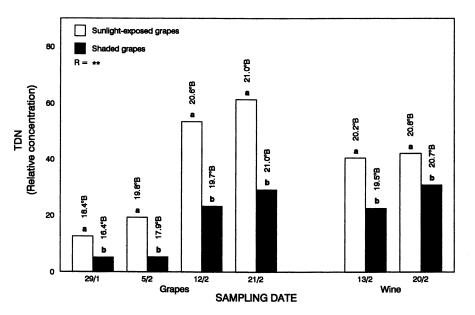


Figure 2. The effect of sunlight, shade and degree of ripeness on the relative concentrations of acid-released 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) in the grapes and wines of Riesling (1991 season). Sugar concentration in Brix (°B) is indicated at each sampling date. R = Level of significance for the increase in TDN concentration in grapes over the sampling period (data for sunlight and shade combined). **Highly significant ($p \le 0.01$). (Reproduced with permission from reference 13. Copyright 1992).

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Norisoprenoid		Grapes				Wine		
		S1 S2 S3 S4 R				<i>S3</i>	<i>S4</i>	
Viti- spirane (trans)	S Sh	6.74a 2.80b	8.99a 2.55b	19.24a 9.32b	25.06a 12.50b	**	22.77a 14.03b	23.60a 15.77b
Viti- spirane (cis)	S Sh	4.94a 1.97b	6.72a 1.79b	15.24a 7.49b	18.67a 11.24a	**	18.86a 12.02b	19.59a 14.05b
Damas- cenone	S Sh	5.62a 5.34a	6.16a 5.26a	6.07a 7.53a	7.10a 7.54a	**	1.98a 2.28a	2.71a 3.18a
Acti- nidol 1	S Sh	1.30a 0.50b	1.69a 0.91b	3.75a 2.01b	3.56a 2.12b	**	4.10a 2.66b	4.58a 3.00b
Acti- nidol 2	S Sh	2.35a 1.15b	2.93a 1.24b	6.97a 4.41a	7.12a 5.00a	**	6.27a 4.14b	6.67a 4.89b
9-HO- mega- stigm- 7-en-3- one	S Sh	1.46a 0.77b	2.23a 1.44b	3.85a 2.27b	3.24a 2.86a	**	2.09a 1.43b	2.93a 2.21b

 Table III. The Effect of Sunlight, Shade and Degree of Ripeness on the

 Relative Concentrations of Acid-released C13-norisoprenoids in Grapes and

 Wines of Riesling

Statistical analysis was performed on log-transformed data. The F-test was followed by the LSD-test. S1-S4 = Sampling stages of grapes; S = Sunlight-exposed grapes; Sh = Shaded grapes.

R = Level of significance for the increase in norisoprenoid concentrations in grapes over the sampling period (data for sunlight and shade combined). **Highly significant (p \leq 0.01).

Values between sunlight-exposed and shaded grapes at each sampling stage designated by the same letter do not differ significantly ($p \le 0.05$).

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Nor	riso-	Grapes					Wine	
prei	noid	S1	S2	S3	S4	R	<i>S3</i>	S4
1	S Sh	11.7a 13.5a	21.0a 19.3a	18.2a 20.6a	14.1a 15.2a	NS	61.0a 65.5a	60.9a 67.2a
2	S Sh	45.4a 31.4b	55.5a 30.5b	67.8a 43.8b	63.9a 47.8b	**	56.5a 39.3b	60.8a 46.6b
3	S Sh	3.6a 1.4b	2.4a 0.8b	2.3a 0.6b	2.4a 7.4b	*	3.3a 1.6b	5.1a 2.6b
4	S Sh	7.0a 5.0b	8.0a 3.6b	8.3a 5.1b	4.2a 4.2a	NS	6.7a 4.2b	6.7a 5.3a
5	S Sh	13.3a 9.3b	17.8a 9.0b	20.3a 11.2a	17.6a 12.7a	**	20.8a 14.9b	20.0a 16.5a
6	S Sh	46.4a 42.2a	85.9a 77.9b	115.4a 119.7b	140.5a 110.6b	**	112.1a 95.7b	133.1a 108.0b
7	S Sh	32.4a 23.2b	69.1a 51.9b	177.3a 128.2b	148.9a 112.2b	**	117.2a 106.2b	139.3a 109.0b
8	S Sh	14.9a 9.9b	22.3a 14.9b	33.4a 18.2b	26.2a 16.8b	**	27.8a 21.6b	31.0a 21.1b

Table IV. The Effect of Sunlight, Shade and Degree of Ripeness on theRelative Concentrations of Enzyme-released C13-norisoprenoids in Grapesand Wines of Riesling

Norisoprenoid Compound: (1) Megastigm-4,8-dien-3,7-dione, (2) 3-Oxo- α -ionol, (3) 3-Hydroxy-7,8-dihydro- β -ionol, (4) 4-Oxo- β -ionol, (5) 4-Oxo-7,8-di-hydro- β -ionol, (6) Grasshopper ketone, (7) Vomifoliol, (8) 7,8-Dihydro-vomifoliol.

Statistical analysis was performed on log-transformed data. The F-test was followed by the LSD-test. S1 - S4 = Sampling stages of grapes; S = Sunlight-exposed grapes; Sh = Shaded grapes. R = Level of significance for the increase in norisoprenoid concentrations in grapes over the sampling period (data for sunlight and shade combined).

NS = Not significant; *Significant ($p \le 0.05$); **Highly significant ($p \le 0.01$).

Values between sunlight-exposed and shaded grapes at each sampling stage designated by the same letter do not differ significantly ($p \le 0.05$).

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The results of this specific investigation indicated that the composition and quality of wine will depend - amongst others – on whether the grapes ripen in sunlight or shady conditions and when the grapes are harvested during the ripening period. Results of recent studies on the effect of canopy microclimate on Sauvignon blanc composition and quality (14) are in agreement with those obtained for Riesling. The above-mentioned factors should be considered when wines with a lower potential to develop TDN and its accompanying kerosene-like aroma, have to be produced.

Wine-aging

The effect of storage time and temperature on free TDN levels and the typical kerosene-like aroma intensity of Riesling wines from three different regions was investigated (15). Wines were stored at 15°C and 30°C, for four and one year at each temperature, respectively. Samples were taken at regular intervals during the aging period for sensory evaluation and chemical analyses.

Significant increases in TDN concentration and kerosene-like aroma intensity were observed for all three wines at both 15°C and 30°C (Figures 3 and 4). Both TDN concentration and the kerosene-like aroma intensity increased at a faster rate at 30°C than at 15°C, and were followed by slight decreases after about 20 weeks of storage at 30°C. This tendency may be ascribed to the transformation of TDN to other components. From these results it is clear that aging plays an important role in the development of TDN and it's accompanying kerosene-like aroma.

Conclusions and Recommendations

Precursors, macroclimate/region, sunlight, shade, grape maturity and storage time and temperature have a pronounced effect on the concentration of TDN and the accompanying negative, kerosene-like aroma intensity of Riesling wines. The levels of carotenoids, glycosidically-bound C_{13} -norisoprenoids, as well as those of free volatiles are related to the degree of exposure to sunlight and shade, to grape maturity, as well as to wine-aging temperatures and time. In order to produce Riesling wines with a lower potential to form TDN, especially in warm climate, wine-producing countries, the following practices can be followed: Cool localities have to be selected for the cultivation of Riesling. The grapes have to be matured in shady/not direct sunlight conditions, and effective canopy management is therefore necessary to obtain an ideal light/shade balance. Harvesting at too high grape maturity should be avoided, especially in warm regions or during warm seasons. To further restrict the formation of TDN during wine-aging to sensorially acceptable levels, wines should be stored under cool conditions, e.g. at temperatures of 15°C or lower.

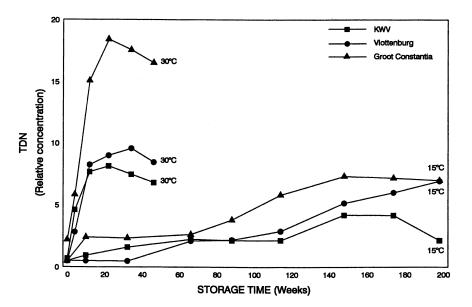


Figure 3. Effect of storage time and temperature on relative TDN concentration in Riesling wines from three regions, namely Durbanville (KWV), Stellenbosch (Vlottenburg) and Constantia (Groot Constantia) (1987 season). (Reproduced with permission from reference 15. Copyright 1992)

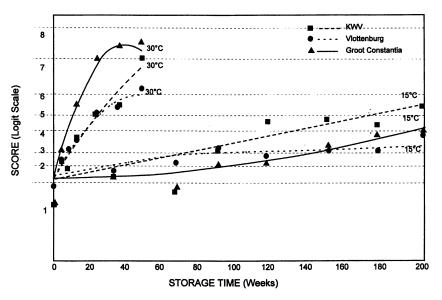


Figure 4. Effect of storage time and temperature on the intensity of the kerosene-like aroma of Riesling wines from three regions, namely Durbanville (KWV), Stellenbosch (Vlottenburg) and Constantia (Groot Constantia) (1987 season). (Reproduced with permission from reference 15. Copyright 1992)

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Chapter 21

Formation of 1,1,6-Trimethyl-1,2-Dihydronaphthalene and Other Norisoprenoids in Wine: Considerations on the Kinetics

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> The norisoprenoids 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), isomeric vitispiranes (VTP) and actinidols, as well as B-damascenone are important aroma contributors in wine. Several free and glycosidically bound C_{13} -polyols have been elucidated as possible progenitors. The recent isolation of a novel important TDN precursor in grape products has been accomplished by our group. Labeling studies indicated a different reaction mechanism as that previously published. Experiments involving acid and isotopic catalysis and the different rates of norisoprenoid formation observed could justify different initial production capabilities and different TDN levels in similarly aged wines. Remarkable differences between precursor contents seem to be correlated to climatically different production areas. A remarkable amount of TDN and VTP precursors has also been found in the so-called free aroma fraction. In order to evaluate the total precursors in grape products (including those of actinidols and B-damascenone) experiments have been performed by direct heattreatment (50°C) of such products at pH 3 with different percentage of D₂O. Bound forms have been reacted only in D₂O. All compounds have been evaluated by HRGC-MS analyses after headspace enrichment using a SPME technique.

TDN (1) and other C_{13} -norisoprenoids like vitispiranes (2), actinidols (3), and β -damascenone (4) (Figure 1) are considered as important aroma contributors in fruits and beverages. Aroma compounds 1-4 are rearrangement products of non-volatile precursors (mainly glycosides) which are present in various parts of the plants (1-3). Model degradation reactions with specific compounds or isolated fractions were performed at different length of time, temperature and pH conditions, followed by different enrichment techniques like solvent extractions, simultaneous distillation-extraction (SDE) or the recently applied headspace solid phase micro-extraction (SPME) (4-7).

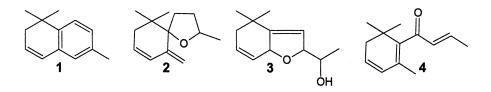


Figure 1. Structures of important volatile C_{13} -norisoprenoids of wines, i.e. TDN (1), vitispiranes (2), actinidols (3), and β -damascenone (4).

A particular role of these compounds for the aroma complexity and intensity during the aging of wines has been demonstrated (8-12). Mechanisms of formation and possible pathways via degradation of different grape carotenoids have been proposed (4,13). This contribution presents results on the chemically induced kinetics of the target volatiles **1-4** - also on the basis of deuterium labeling studies - in order to indicate some important factors determining their formation and to evaluate the existence of different precursors. A better understanding of the hydrolytic conversion of precursor substances will allow an improved prediction of wine quality evolution.

1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN)

TDN is one of the most typical contributors to the aroma of aged Rhine Riesling wines produced from warmer growing areas. It can also be detected at lower levels in Riesling crossings, such as, e.g., Kerner (*Riesling x Trollinger*). In concentrations above 20 ppb, TDN causes an unpleasant kerosene or petrollike note (8, 14). Sensory and analytical studies carried out with German Riesling wines indicated a positive influence on Riesling aroma when TDN was present at a concentration of approximately one fourth of its threshold level (15).

Multiple precursors to TDN have been identified in grapes and wines, being mainly present in glycosidically bound form. Structures involved in TDN formation included 3,6-dihydroxy-7,8-dihydro-ß-ionone (5a) that is in equilibrium with 2,6,10,10-tetramethyl-1-oxaspiro[4.5]dec-6-ene-2,8-diol (5b). Further precursors are 3.4-dihydroxy-5-megastigmen-9-one (5c) and 4,6dihydroxy-4-megastigmen-9-one (6) the latter being O-substituted in position 4 (for details cf. refs. 16-19). Precursor fractions were obtained by XAD-2 adsorption of Rhine Riesling musts and reacted in D₂O at 50°C using different pH values from 2.0 to 3.5. In these experiments two different TDN production kinetics were observed, a fast one - likely corresponding to a 1st order-type reaction - giving rise to a formation of TDN 1, and a slower one, producing 1,1-dimethyl-6-deuteromethyl-1,2-dihydro-7-deuteronaphthalene (TDN- d_4 , 8). At the pH values employed, TDN 1 is considered to be generated from glucoconjugates 5a-c, while TDN-d₄ 8 is likely to be derived from compound 6 (cf. Figure 2) (19).

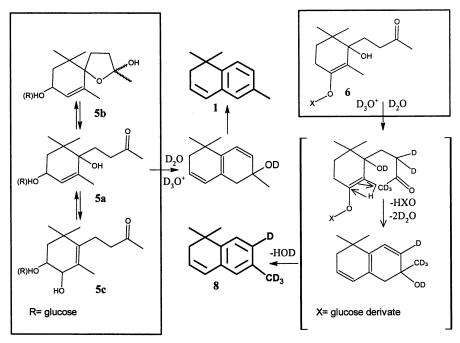


Figure 2: Proposed schematic pathway of TDN and TDN- d_4 formation in D_2O/D_3O^+ .

In Carotenoid-Derived Aroma Compounds; Winterhalter, P., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2001. A different combination of both kinetics could justify the evolution curves shown in Figure 3 (20). TDN 1 and TDN-d₄ 8 formation reaches a maximum followed by a further degradation which is more prominent at lower pH levels (6). This effect can also be observed in the case of vitispirane 2 formation and seems not to be linked to oxidative storage conditions, at least for the reaction directly performed in wine medium (21).

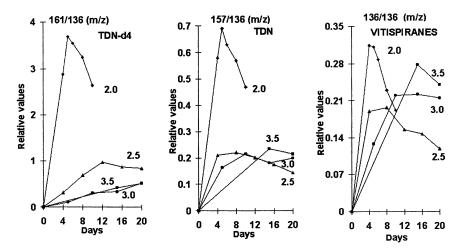


Figure 3: Kinetics of norisoprenoid formation from glycoconjugates of a younger North Italian Rhine Riesling wine in D₂O at pH values 2 - 3.5 (50 °C; relative values as ratios of specific m/z ions to m/z 136 of naphthalene-d₈ as internal standard; adapted from ref. 6).

The remarkably lower maximum level for both TDN forms, especially when passing from pH 2 to pH 2.5, could indicate the existence of common reaction steps in both mechanisms that may be of different relevance at these two pH values (cf. Figure 3) (6). Acid catalysis is evident throughout the different pH values examined. Temperature also seems to play an important role for the rate of TDN formation (20). This was obvious from an experiment in which three Riesling wines were submitted to prolonged storage at 15°C and 30°C, respectively. The maximum yield of TDN, that was reached at 30°C after about 20 weeks, was at least twice higher compared to the maximum of the 15°C sample. In the latter case the maximum was observed after 150-180 weeks. This indicates that at higher temperature another rate-determining step could become competitive so that other intermediates (e.g. Riesling acetal) can become more important for the final equilibrium (17,18).

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A significant amount of TDN precursors was also found in the free aroma fraction isolated from wines after XAD-2 adsorption and elution by pentane/dichloromethane (2:1, v/v). This fraction was also reacted under the same conditions (pH 2.5, 50°C, 10 days heating) as described for the bound forms (6). Older Sicilian wines have been found to be about 5-10 fold richer in TDN compared to younger South African and North Italian wines. In one year old wines, the total TDN level (i.e. the sum of free and thermally generated TDN) in the free aroma fraction was significantly higher (up to ten times !) than in the bound fraction. This indicates that a steady hydrolysis of progenitors is in progress and a complete evaluation of norisoprenoid formation has to include also the precursors which are present in the free aroma fraction. In the abovementioned wines, precursors generating deuterium-labeled TDN (TDN-d) were about 3 to 20 times more abundant than those yielding non-labeled TDN.

By analyzing northern and southern Italian as well as South African wines differences in the content of TDN and TDN-d₄ generating precursors were observed (6,14). Importantly, the southern Italian as well as the South African wines yielded 2-3 times more of non-labeled TDN. In addition, a remarkably different developing rate of TDN in some German and North American Riesling wines (even with an opposite ratio of TDN/TDN-d₄ precursors) indicates different kinetics for the hydrolysis of the precursors which may be linked to particular climatic situations. On the other hand, no significant influence of different glycosidase activities of wine yeasts on the rate of TDN formation seems to be evident (15). The kinetics of TDN formation has been further investigated in order to understand possible isotopic effects which could speed up the reaction itself (22) (cf. Figure 4).

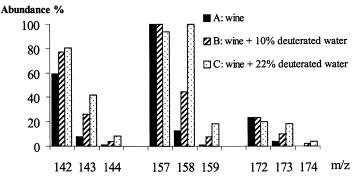


Figure 4: Variation of relative abundance of some MS fragments (m/z) as markers of TDN and its deuterated forms in a North Italian 1998 Müller-Thurgau wine and D₂O-diluted samples (pH 3; 50°C; 15 days).

In Carotenoid-Derived Aroma Compounds; Winterhalter, P., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

Thus, Riesling wines - including a Riesling crossing (Müller-Thurgau) were diluted with different amounts of deuterated water. After heat-treatment (50°C) at pH 3 the volatiles were analyzed with SPME. As a result no apparent increase of the reaction rate was observed for the diluted samples. It was, however, evident that the TDN-d₁ to TDN ratios (determined by ratios of m/z173 to 172 or m/z 158 to 157) varied considerably (up to sevenfold increase as can be seen from Figure 4). If one takes into account the different deuteration probabilities, deuteration at C-7 or at the methyl group at C-6 is likely to support the observed relative levels of the fragments m/z 157 and 158 with respect to m/z 142 as well as the results for a higher deuteration rate of TDN. The observed increase in labeled TDN is obviously the result of a positive isotope effect due to H⁺-catalysis that favors the formation of labeled species even in partially deuterated water as it is known for a keto-enolic equilibrium reaction. However, in the case of the TDN production mechanism, this step appears not to be rate-determining as it is demonstrated by identical shape of the kinetics curve for m/z 172 (molecular ion of TDN) with respect to other labeled fragments in wine that has been diluted (3.5:1, vv) with D₂O. Furthermore, the same kinetics observed for the fragment m/z 172 demonstrate that non-labeled TDN is mainly produced from the same precursor as its deuterated forms. Different findings were made for an Australian Riesling wine (cf. Figure 5).

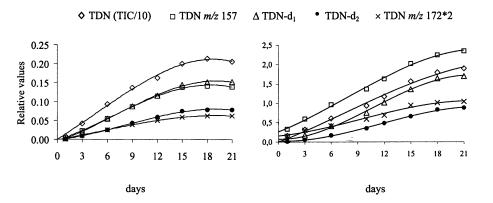


Figure 5: Formation kinetics of TDN and its deuterated forms monitored through quantification of MS specific m/z ions with 2-octanol (TIC) as internal standard (wine + 22% D₂O, pH 3; 50°C). Left: North Italian 1998 Müller-Thurgau; right: Australian (Barossa valley) 1999 Rhine Riesling.

In Carotenoid-Derived Aroma Compounds; Winterhalter, P., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2001. In an attempt to clarify the nature of the two different precursor types in Italian and Australian wine, respectively, the wine residues (after evaporation of ethanol and lyophilization) were similarly heat-treated in D_2O . The production of TDN from the bound precursor forms which are still present in the residues was remarkably lower than that obtained by heat-treatment (50°C) of the original wines. A prevalence of the tetradeuterated form with respect to the non-deuterated form of TDN or *vice versa* confirmed what had been observed by reacting the whole wines as well as two different formation kinetics (Figure 6).

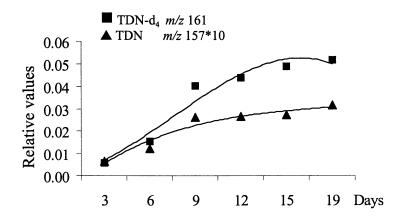


Figure 6: Formation kinetics in D_2O of TDN and TDN-d₄, generated by heating bound precursor forms present in wine residues (pH 3; 50°C, relative values to internal standard).

The possible role of grape ripeness on the ratio of the two types of TDN precursors has also been studied. Rhine Riesling musts were prepared from grapes at different stages of ripeness and heat-treated (50°C) after dilution with D_2O (3.5 : 1, vv). In these experiments, no influence of grape ripeness on TDN ratios was apparent.

Finally, by performing SPME analysis on differently aged wines, a novel TDN derivative has been tentatively identified on the basis of its MS fragmentation as 1,1,6-trimethyl-1,2,5,6-tetrahydro-6-ethoxynaphthalene. MS-data (70 eV) m/z 218(9), 203(12), 189(1), 173(5), 172(41), 157(79), 143(17), 142(13), 132(39), 117(13), 115(21), 91(16), 73(20), 69(14), 55(28), 45(25), 43(100). The compound showed a formation kinetic superimposed to that of TDN. The presence of this ether even at about only one twentieth of the concentration of TDN in the headspace of wine could be of possible sensorial relevance.

Vitispiranes

Isomeric compounds 2 (cf. Figure 1) are known to contribute an eucalyptus-like/balsamic nuance to wine flavor when present at a level of approximately 800 μ g/L (8). This level is only reached in older Riesling wines, and also in some non-floral varieties. In some cases the concentration of vitispiranes may not be higher than 30 ppb, like, e.g., in Italian Chianti (23) or Recioto di Soave (5). Still, the balsamic/resinous scent, giving complexity to the flavor of the latter wines, can roughly be correlated to the vitispiranes content as well as additional norisoprenoid compounds, such as, e.g. isomeric actinidols. Like in the case of TDN formation, multiple precursors to these compounds are present in grapes and wine (24). Bound forms of megastigm-5-ene-3,4,9-triol and of isomeric 3-hydroxytheaspiranes have been identified (25) together with glycosides of 1-(3-hydroxybutyl)-6,6-dimethyl-2-methylen-3-cyclohexen-1-ol (26).

Similar investigations as described for TDN with regard to the influence of pH on the kinetics of formation of non-labeled, mono- and dideuterated vitispiranes were carried out. For the heat-treatment (50°C) in D₂O glycosidic fractions of Riesling must or wine were employed. Our data showed (i) first order-like kinetics with a generally higher vitispirane formation rate compared to TDN, (ii) no effect of pH on the final concentration of the non-deuterated form (cf. Figure 3), and (iii) an H⁺-catalysis effect as expected for cyclisation/dehydration reactions (Figure 7) (6).

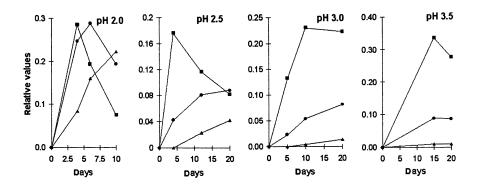


Figure 7: Formation kinetics in D₂O of vitispiranes (VTP) as well as deuterated forms from precursor fractions monitored through specific MS fragments ratios (VTP: 192/136; ● VTP-d₁:193/136; ▲ VTP-d₂:194/136; wine type, reaction conditions and reference as outlined in Figure 3).

The generation of various deuterated vitispirane species can be explained by acid-catalyzed degradation of multiple known precursors substances, taking into account the possible addition and elimination reactions of D_2O in various positions of the megastigmane ring. Also for vitispiranes, a formation from progenitors in the free aroma fraction was observed. Based on deuterium labeling remarkable differences for the precursors among Riesling wines from different production areas, e.g., South Africa or different regions in Italy, was observed (6). With the original wines, at pH 3 and 50°C, no clear positive isotope effect could be observed. However, by reacting the wine residues in D_2O at 50°C, different kinetics of formation could be confirmed for the nondeuterated and deuterated compounds (cf. Figure 8).

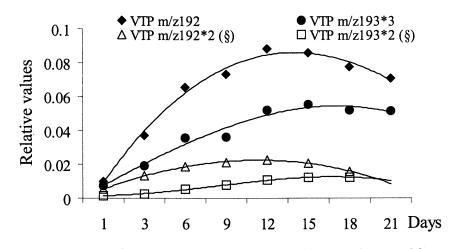


Figure 8: Kinetics of formation of VTP (m/z=192) and its monodeuterated form (m/z=193) produced by heating both wine and wine residue (§) (wine + 22% D_2O ; residue in D_2O ; pH 3; 50°C).

Actinidols

Little information is available concerning the formation of isomeric actinidols (3) in wines as well as their sensorial contribution to wine aroma (20). The odor of actinidols has been described as camphoraceous (27). In our own

In Carotenoid-Derived Aroma Compounds; Winterhalter, P., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2001. studies effluent sniffing at the elution times of isomeric 4 in GC analyses of extracts from thermally-treated grape glycosides revealed a woody-resinous note. Of the four norisoprenoids 1-4 discussed in this chapter, actinidols represent the most abundant C_{13} -compounds among the precursor-derived volatiles. This is also true for aged wine of different varieties, except for Riesling (2,12,23). Formation of actinidols is related to free and bound norisoprenoid triols which are also present in Riesling vine leaves (16,28). Glycosidic precursors of Chardonnay wine were reacted at pH 2.5 and 3, respectively, and the kinetic of formation of the two main actinidol isomers was determined. Although higher amounts of actinidols are formed and no degradation after 5 days takes place, the curves resemble those observed for vitispiranes (Figure 9).

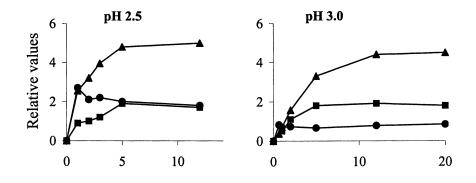


Figure 9: Formation kinetics of C_{13} volatiles hydrolytically liberated from the bound precursor fraction of a Chardonnay must (H_2O ; 50°C). \blacksquare vitispiranes (m/z = 192 x10), \blacktriangle actinidol isomer 1 (TIC/2 at pH 2.5 and TIC at pH 3), and $\bigcirc \beta$ -damascenone (m/z=69).

Using headspace SPME analysis, the ethoxy isomers of actinidols have been tentatively identified. MS data: 164 (18), 163 (90), 145 (17), 135 (9), 121(18), 105 (16), 77 (13), 73 (25), 65 (9), 45 (100). In the headspace analysis, these ethers were more abundant than the actinidols. Moreover, the generation of both ethers has been followed, revealing a steady increase during the examined reaction period. There was no positive isotopic effect apparent (Figure 10).

> In Carotenoid-Derived Aroma Compounds; Winterhalter, P., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

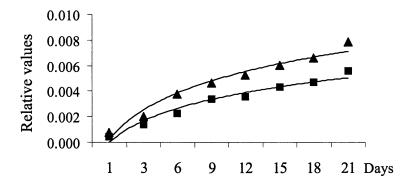


Figure 10: Formation kinetics of actinidol ethyl ethers (m/z163, \blacktriangle isomer 1 and \blacksquare isomer2) in the wine residue $+D_2O$ (pH 3; 50°C).

B-Damascenone

Multiple precursors to β -damascenone (4) are present in wine (24). Both, the 3- and 9-O-glucoconjugate of 3-hydroxy-7,8-didehydro-ß-ionol (9,10) have been identified in Riesling wine (29,30). The 9-O-glucoconjugate 10 was found as the main progenitor of flavor compound 4 (cf. Figure 11). The significance of ketone 4 for wine flavor is discussed controversially (7, 10). B-Damascenone 4 is always accompanied by the odorless 3-hydroxy- β -damascone (11). The ratio between 4 and 11 is usually 1 : 9 (31,32). Bound progenitors were isolated from Chardonnay musts by XAD-2 adsorption and reacted at pH 3 at 50°C in water. The maximum level of B-damascenone was reached within just one day with successive limited variations in concentration during two weeks of heating. The high reactivity of the precursors corresponds with previous results (7). By performing the reaction at pH 2.5, the same kinetics is observed, but the maximum level was nearly twice as high. In both cases a 1st order reaction seems to be the dominant formation process (cf. Figure 9). Variations in concentration due to pH changes are most likely caused by the presence of different precursors (free and bound forms, cf. Figure 11), which are expected to show different reactivities (33). On the other hand, β -damascenone reaches its maximum level in wine already within one year of aging (7). In fact, the concentration of B-damascenone measured by headspace SPME during the heating (50°C) of a Müller-Thurgau wine at pH 3 maintained a constant value (Figure 12).

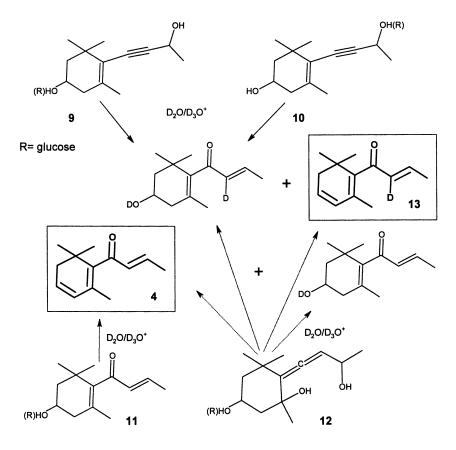


Figure 11: Proposed pathways of β -damascenone formation and a monodeuterated form (13) in D_2O .

Addition of D_2O to the wine (1 : 3.5, vv) increased the intensity of the MSfragment at m/z 70 relative to m/z 69. This fact seems to exclude a positive isotope effect in a certain reaction step, but supports the formation of labeled β damascenone 13. To investigate the kinetics of β -damascenone formation from the bound precursor forms in wine, lyophilized wine was also reacted in D_2O . The formation curves of both non- and monodeuterated β -damascenone are also presented in Figure 12. It is obvious that generation of 4 and 13 from precursors

> In Carotenoid-Derived Aroma Compounds; Winterhalter, P., et al.; ACS Symposium Series; American Chemical Society: Washington, DC, 2001.

in the wine residue proceeds by different kinetics compared to must. Likely precursor candidates are the allenic triol 12 and/or its respective glycoconjugates (32). The observed rates of formation of β -damascenone from the lyophilisate are much lower as in the original wine where β -damascenone can be expected to be derived from the acetylenic precursors 9 and 10.

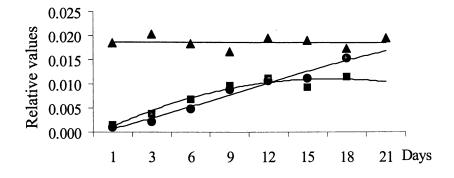


Figure 12: Kinetics of β -damascenone formation in wine (m/z 69, \blacktriangle) and of β damascenone (m/z 69, \blacksquare) and monodeuterated isomer 13 (m/z 70, \bullet) in the wine lyophilisate (+D₂O, pH 3, 50°C).

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