

3,5,7-trimethyl-1,2,4,6-tetrathiepane, 115421-49-3; 4,7-dimethyl-1,2,3,5,6-pentathiepane, 101517-94-6.

LITERATURE CITED

- Boelens, M.; Van der Linde, L. M.; De Valois, P. J.; Van Dort, H. M.; Takken, H. C. "Organic Sulfur Compounds from Fatty Aldehydes, Hydrogen Sulfide, Thiols, and Ammonia as Flavor Constituents". *J. Agric. Food Chem.* 1974, 22, 1071-1076.
- Brinkman, H. W.; Copier, H.; de Leuw, J. J. M.; Tjan, S. B. "Components Contributing to Beef Flavor: Analysis of the Headspace Volatiles of Beef Broth". *J. Agric. Food Chem.* 1972, 20, 177-181.
- Buttery, R. G.; Seifert, R. M.; Ling, L. C. "Characterization of Some Volatile Potato Components". *J. Agric. Food Chem.* 1970, 18, 538-539.
- Buttery, R. G.; Ling, L. C.; Teranishi, R.; Mon, T. R. "Roasted Lamb Fat: Basic Volatile Components". *J. Agric. Food Chem.* 1977, 25, 1227-1229.
- Chang, S. S.; Hirai, C.; Reddy, B. R.; Herz, K. O.; Kato, A.; Sipma, G. "Isolation and Identification of 2,4,5-Trimethyl-3-oxazoline and 2,5-Dimethyl-1,2,4-trithiolane in the Volatile Flavor Compounds of Boiled Beef". *Chem. Ind.* 1968, 1639-1640.
- Chen, C. C.; Ho, C.-T. "Identification of Sulfurous Compounds of Shiitake Mushroom (*Lentinus edodes* Sing.)". *J. Agric. Food Chem.* 1986, 34, 830-833.
- Fors, S. "Sensory Properties of Volatile Maillard Reaction Products and Related Compounds". In *The Maillard Reaction in Foods and Nutrition*; Waller, G. R., Feather, M. S., Eds.; ACS Symposium Series 215; American Chemical Society: Washington, DC, 1983; pp 185-286.
- Heller, S. R.; Milne, G. W. A. *EPA/NIH Mass Spectra Data Base*; U.S. Government Printing Office: Washington, DC, 1978.
- Horvat, R. J. "Identification of Some Volatile Compounds in Cooked Chicken". *J. Agric. Food Chem.* 1976, 24, 953-958.
- Kawai, T.; Irie, M.; Sakaguchi, M. "Degradation of 2,4,6-Trialkyltetrahydro-1,3,5-thiadiazines during Storage". *J. Agric. Food Chem.* 1985, 33, 393-397.
- Kleipool, R. J. C.; Tas, A. C. "Products of the Maillard. III. 3-Methyl-1,2,4-trithion". *Riechst. Aromen, Koerperpflgem.* 1974, 7, 204-207.
- Kubota, K.; Kobayashi, A.; Yamanishi, T. "Some Sulfur Containing Compounds in Cooked Odor Concentrate from Boiled Antarctic Krills (*Euphausia superba* Dara.)". *Agric. Biol. Chem.* 1980, 44, 2677-2682.
- MacLeod, G. "The Scientific and Technological Basis of Meat Flavours". In *Developments in Food Flavours*; Birch, G. G., Lindley, M. G., Eds.; Elsevier Applied Science: London, 1987.
- Majlat, P.; Erdos, Z.; Takacs, J. "Calculation and Application of Retention Indices in Programmed Temperature Gas Chromatography". *J. Chromatogr.* 1974, 91, 89-110.
- Mecchi, E. P.; Pippen, E. L.; Lineweaver, H. "Origin of Hydrogen Sulfide in Heated Chicken Muscle". *J. Food Sci.* 1964, 29, 393-399.
- Nixon, L. N.; Wong, E.; Johnson, C. B.; Birch, E. J. "Nonacidic Constituents of Volatiles from Cooked Mutton". *J. Agric. Food Chem.* 1979, 27, 355-359.
- Nursten, H. E. "Aroma Compounds from the Maillard Reaction". In *Developments in Food Flavours*; Birch, G. G., Lindley, M. G., Eds.; Elsevier Applied Science: London, 1987.
- Ohloff, G.; Flament, I. "Heterocyclic Constituents of Meat Aroma". *Heterocycles* 1978, 2, 663-695.
- Ohloff, G.; Flament, I.; Pickenhagen, W. "Flavor Chemistry". *Food Rev. Int.* 1985, 1, 99-148.
- Pippen, E. L.; Mecchi, E. P. "Hydrogen Sulfide, a Direct and Potentially Indirect Contributor to Cooked Chicken Aroma". *J. Food Sci.* 1969, 34, 443-446.
- Shu, C.-K.; Hagedorn, M. L.; Mookherjee, B. D.; Ho, C.-T. "pH Effect on the Volatile Components in the Thermal Degradation of Cysteine". *J. Agric. Food Chem.* 1985, 33, 442-446.
- Tang, J.; Jin, Q. Z.; Shen, G. H.; Ho, C.-T.; Chang, S. S. "Isolation and Identification of Volatile Compounds from Fried Chicken". *J. Agric. Food Chem.* 1983, 31, 1287-1292.
- Ten Noever de Brauw, M. C.; Bouwman, J.; Tas, A. C.; La Vos, G. F. *Compilation of Mass Spectra of Volatile Components in Foods*; Central Institute for Nutrition & Food Research: Zeist, 1983.
- Vitzthum, O. G.; Werkhoff, P. "Oxazoles and Thiazoles in Coffee Aroma". *J. Food Sci.* 1974, 39, 1210-1215.
- Wilson, R. A.; Katz, I. "Review of Literature on Chicken Flavor and Report of Isolation of Several New Chicken Flavor Components from Aqueous Cooked Chicken Broth". *J. Agric. Food Chem.* 1972, 20, 741-747.

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Original Composition of Marjoram Flavor and Its Changes during Processing

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The composition of monoterpenes in essential oils or flavor extracts from marjoram (*Majorana hortensis* Moench) depends on the method applied for their isolation. Experiments with solvent extracts, with leaf material ground under liquid nitrogen, and with direct injections of the content of oil glands revealed the presence of a common precursor structure. The study of rearrangement reactions of sabinene hydrate and sabinene hydrate acetate confirmed that the sabinene hydrate skeleton represents the primary monoterpene structure in marjoram. Two activated forms are responsible for the formation of rearranged monoterpenes during workup of the plant material: a "bound" form, presumably (*Z*)-sabinene hydrate pyrophosphate, and a "free" form, (*Z*)-sabinene hydrate acetate, which is the main component in the oil glands together with (*Z*)-sabinene hydrate.

Marjoram is a spice plant used because of its content of so-called "essential oil". In the food industry the distilled oil frequently comes to application, on account of

the greater storage stability, the missing microbial contamination, and the known content of individual compounds. The term essential oil implicates by definition the method of preparation, namely the separation of steam-volatile substances by distillation at atmospheric pressure.

The usual gas chromatographic analysis of flavor compounds of such herbs or spices mostly starts with the

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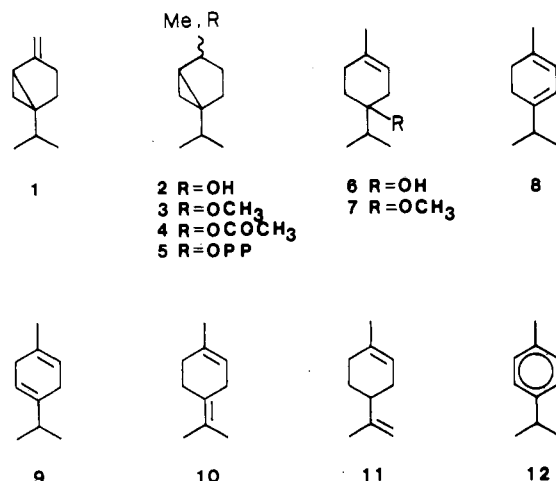


Figure 1. Monoterpenes referred to in this work: 1, sabinene; 2, (*E/Z*)-sabinene hydrate; 3, (*E/Z*)-sabinene hydrate methyl ether; 4, (*Z*)-sabinene hydrate acetate; 5, (*Z*)-sabinene hydrate pyrophosphate; 6, terpinen-4-ol; 7, terpinen-4-ol methyl ether; 8, α -terpinene; 9, γ -terpinene; 10, terpinolene; 11, limonene; 12, *p*-cymene.

preparation of an essential oil, particularly since it implies that the performance of the chromatographic system is not disturbed by nonvolatile substances.

The application of elevated temperatures for the distillation however involves the risk of chemical changes of the original plant components. Such changes, especially of terpenes, have been reported in the literature, e.g., formation of terpinen-4-ol (6), terpinolene (10), and α - (8) and γ -terpinene (9) from sabinene (1) and sabinene hydrate (2) (Koedam and Looman, 1980). The flavor compounds of marjoram also mainly consist of monoterpenes; the latest reports about marjoram essential oils [see Nykänen (1986) and references cited herein] always refer to (*Z*)-sabinene hydrate (2)—possessing a smell reminiscent of marjoram (Lossner, 1967, 1968)—and terpinen-4-ol (6) as main components in varying amounts. For this reason even the occurrence of different chemotypes of marjoram was discussed, a sabinene hydrate type and a terpinen-4-ol type (Granger et al., 1975). The formulas of the major monoterpenes of marjoram essential oils are shown in Figure 1.

Our earlier work demonstrated that the method of isolation of flavor compounds has a great influence on the qualitative and quantitative composition of the extracts (Nitz et al., 1985). It also appeared that there exists a type of bound reactive precursor that can be isolated by means of a RP-18 column, analogously to the isolation of terpene glycosides (Williams et al., 1982). This precursor hydrolyzes spontaneously upon addition of water, liberating a number of components of marjoram essential oils, among them monoterpene alcohols and hydrocarbons.

This hydrolysis occurs at room temperature and without acid catalysis; therefore, it can hardly be attributed to possible glycosidically bound forms of flavor compounds, which are encountered frequently in plants (Hörster, 1979; v.d. Gathen and Hörster, 1979).

Further attempts to characterize this reactive precursor compound and the nature of changes of marjoram flavor components during processing of the plant material are reported.

EXPERIMENTAL SECTION

Materials. Fresh, deep-frozen, and dried marjoram (*Majorana hortensis* Moench) from experimental cultivation in Erdinger Moos, FRG, was used.

Preparation of Extracts. A 200-g portion of marjoram leaf material was preextracted four times with 250 mL of pentane/dichloromethane (2:1, v/v) each to remove free flavor compounds; solvent was decanted after 20 min of stirring; residual solvent was blown off under a stream of nitrogen; and the leaves were vacuum-dried at room temperature for 2 h.

Ethanol Extract (A). A 30-g sample of preextracted material was further extracted with 150 mL of EtOH for 5 h on a shaking machine, and the solution was filtered off and taken to dryness under vacuum in a rotary evaporator. The residue was stored in a desiccator until use.

Acetone Extract (B). Of the preextracted material 100 g was extracted with 2 L of acetone as above.

***n*-PrOH/NH₄HCO₃ Extract (C).** A 30-g sample of preextracted material was further extracted with 500 mL of 0.1 N aqueous NH₄HCO₃/1-propanol (1:4, v/v) as above.

Hydrolysis of Extracts. Spontaneous Hydrolysis. To about 20 mg of extract A or B was added 50 mL of pentane and the resultant suspension stirred for 10 min. The pentane phase was decanted, residual solvent blown off under a stream of N₂, and 50 mL of water added. After 2 min of stirring, the solution was extracted three times with 60 mL of ether each time.

Acid Hydrolysis. The procedure was as above, except for addition of a mixture of 50 mL of water and 5 mL of 0.1 N H₂SO₄.

Methanolysis. A 50-mg sample of extract B (dry) was extracted three times with a total of 60 mL of pentane. The pentane was removed, and after addition of 20 mL of MeOH the sample was refluxed for 1 h. After being allowed to cool to room temperature, the sample was diluted with water to an alcohol content of about 10% and extracted three times with pentane.

Reductive Cleavage with Sodium Borohydride. One-tenth of extract C was dissolved in EtOH, and one tablet sodium hydroxide and 0.5 g of sodium borohydride were added. When the gas evolution stopped, the solution was diluted with water to 20% alcohol and extracted three times with pentane.

Determination of Phosphate after Cleavage. To the aqueous-alcoholic solution was added a few milliliters of saturated BaCl₂ solution after extraction of liberated flavor compounds with pentane. The solution was reduced in a rotary evaporator to approximately 10 mL; the white precipitate was filtered off, washed with water, and dissolved in 1 N HCl; and the solution was reduced to about 2 mL and evaporated with concentrated nitric acid on the Bunsen burner until no more nitrous gas evolved. After cooling, the dissolved residue was taken into 50 mL of water and the phosphate content was determined with a Merck Phosphate quick-test set (molybdate-vanadate color reaction) using a calibrated color chart.

Trituration of Marjoram Material. Samples of 20 g of dried and 10 g of freshly deep-frozen marjoram were desiccated under vacuum at room temperature for 6 h. The stems were picked out, and the remaining leaf material was stored until needed. Of the dried material 0.5 g was poured in a mortar with N₂(l), 0.5 g of anhydrous sodium carbonate added, and the material ground with a pestle, with repeated addition of N₂(l). Then, 20 mL of pentane was added to the powder and the extraction carried out with stirring for 3 min. After decantation of the pentane extract, the usual workup for GC analysis followed.

Synthesis of Sabinene Hydrates. Synthesis was according to the oxymercuration-demercuration procedure described by Brown and Geoghegan (1967). Mercury(II) acetate (0.99 g) was dissolved in 3.1 mL of water and 3.1

mL of THF, and 1 was dropwise added (0.5 mL, 96%, Roth, Karlsruhe, FRG) with 10 min of stirring. Addition of 3.1 mL of 3 M aqueous sodium hydroxide solution and dropwise addition of 3.1 mL of 0.5 M NaBH₄ in 3 M NaOH followed. A few milliliters of saturated solution of NaCl were added, and the THF phase was separated by means of a pipet. After dilution with water to the 4-fold volume, it was extracted with 3 × 5 mL of pentane. The extract contained in 95% yield a mixture of (*Z*)-2 and (*E*)-2 (42:58). Preparative separation of the stereoisomers was achieved by column chromatography on silica using a pentane-ether gradient.

Synthesis of Sabinene Hydrate Acetate. (*Z*)-2 (80 mg) was acetylated in a Teflon-sealed tube with 0.5 mL of acetic anhydride in 0.5 mL of pyridine with addition of a small amount of anhydrous sodium sulfate to absorb the reaction water. The tube was heated for 1 h to 120 °C; after cooling, the mixture was diluted with water, the acetate extracted with pentane, and the organic phase dried and concentrated. Subsequent chromatographic purification used flash chromatography (Still, 1978) on silica and a pentane-ether gradient. Structural characterization of 4 using ¹H NMR, ¹³C NMR, MS, and IR was made by C. Pollter during the course of her Diploma Thesis (Pollter, 1986).

Rearrangement Reactions of Sabinene Hydrate (2) and Sabinene Hydrate Acetate (4). *Acid-Catalyzed Rearrangement of 2.* Of the synthesized sabinene hydrate mixture, 1 drop was dissolved in 50 mL of water, a few milliliters of 0.1 N H₂SO₄ were added, and the water phase was covered with a layer of pentane (20 mL). After 16 h at room temperature the water phase was extracted with the pentane and the extraction repeated twice (10 mL of pentane each).

Thermal Rearrangement of 2. Of the synthesized sabinene hydrate mixture, 1 drop was dissolved in 30 mL of water and the resultant mixture refluxed for 1 h. After cooling, the water phase was extracted with pentane.

Thermal Rearrangement of 4. The pentane extract of 1 g of marjoram (stirred for 20 h in aqueous buffer (pH 7) under a layer of pentane at room temperature), containing 34% (*Z*)-2, was taken to dryness under a stream of N₂. Thirty milliliters of water was added, and the resultant mixture was refluxed for 30 min. After cooling, the water phase was extracted with pentane.

Methanolysis of 4. To 10 mg of 4 was added 20 mL of MeOH and the mixture refluxed 30 min. After being cooled and diluted with water to about 20% alcohol, the mixture was extracted with pentane.

Workup of Solvent Extracts for Gas Chromatographic Analysis. Solvent extracts were dried with anhydrous sodium sulfate, filtered, and concentrated to a definite volume (usually 1 mL) in a water bath at 40 °C on a Vigreux column. For quantitative determination, a definite amount of standard was added (2-decanol or 2-dodecanol).

Gas Chromatography and Gas Chromatography-Mass Spectrometry. The Becker Packard Model 417 GC (FID) was modified for capillary columns with a Carlo Erba injector with split inlet: column, 30 m × 0.32 mm SE-54 (film 0.15 μm), chemically bonded and cross-linked; temperature program, 60 °C (5 min) to 250 °C (2 °C/min); carrier gas, H₂. The Carlo Erba Fractovap 2300 GC (FID) was equipped with a capillary column, 25 m × 0.3 mm OV-1 (film 0.5 μm), cross-linked; temperature program, 60 °C (3 min) to 200 °C (2 °C/min); carrier gas, H₂; quantitative evaluation made with internal standard; integrator, Spectra Physics SP 4000; GC-MS, Finnigan 1020 (quad-

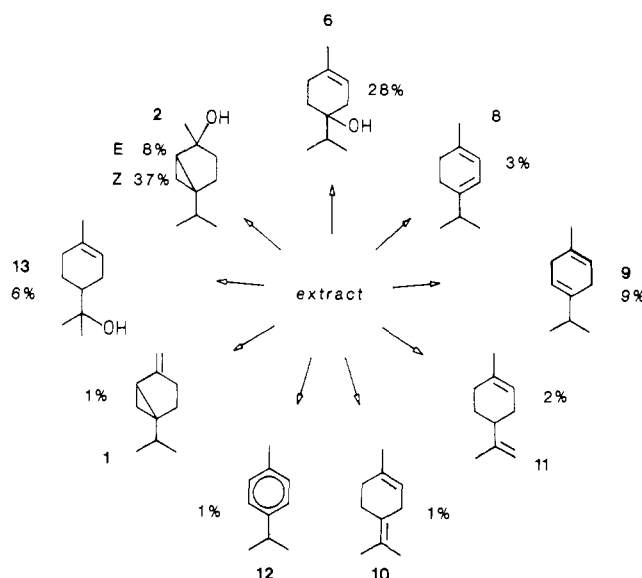


Figure 2. Distribution of monoterpenes obtained by spontaneous hydrolysis of acetone extract (B): 1-12, see Figure 1; 13, α -terpineol.

rupole). The Perkin-Elmer Sigma 3 GC was equipped with a capillary column, fused silica 30 m × 0.25 mm DB-5 (film 0.25 μm); temperature program, 60 °C (5 min) to 250 °C (2 °C/min); carrier gas, He; ionization energy, 70 eV; IncoS data system, NBS Library.

Chemicals and Reagents. All chemicals were of p.a. quality from trade; solvents were additionally distilled in silver-coated packed columns.

RESULTS AND DISCUSSION

Observation of the aforementioned spontaneous hydrolysis of the RP-18 isolate (Nitz et al., 1985) prompted us to extract marjoram with organic solvents in order to reduce the degree of hydrolysis during workup. Therefore, extracts were prepared with ethanol (A) and acetone (B) as solvents, taken to dryness, and treated with pentane several times to remove free flavor compounds.

The distribution of monoterpenes obtained after treatment of the acetone extract with water at room temperature is shown in Figure 2. Similar results were obtained by hydrolysis of the ethanol extract, with the reservation that the latter extract contained 11% (*Z*)-sabinene hydrate acetate (4); the special role of this ester will be discussed later.

The pattern of monoterpenes is—in a qualitative view—very similar to that of conventionally prepared essential oils (Nykänen, 1986), despite the fact that in the above solvent extracts nearly no free terpenes should be present after pretreatment with pentane. The absence of free flavor compounds in considerable amounts could easily be confirmed by the nose: the dry extract itself has no or only little smell; when water was added, a spicy and marjoramlike smell appeared. Acid hydrolysis of these extracts expectedly increased the portion of rearranged monoterpenes, with terpinen-4-ol (6) dominating (52%), followed by γ - (9) and α -terpinene (8) (21% and 8%). The portions of sabinene hydrates were 5% (*Z*) and 3% (*E*) (2).

These rearrangement reactions occur almost spontaneously at room temperature; so reactions of free sabinene hydrate or sabinene, described in literature (Taskinen, 1976; Granger et al., 1975; Koedam and Looman, 1980), can be ruled out, because all reactions reported are based on the application of heat and/or acid.

Methanolysis of these extracts produced the same methyl ethers as previously observed in equally treated

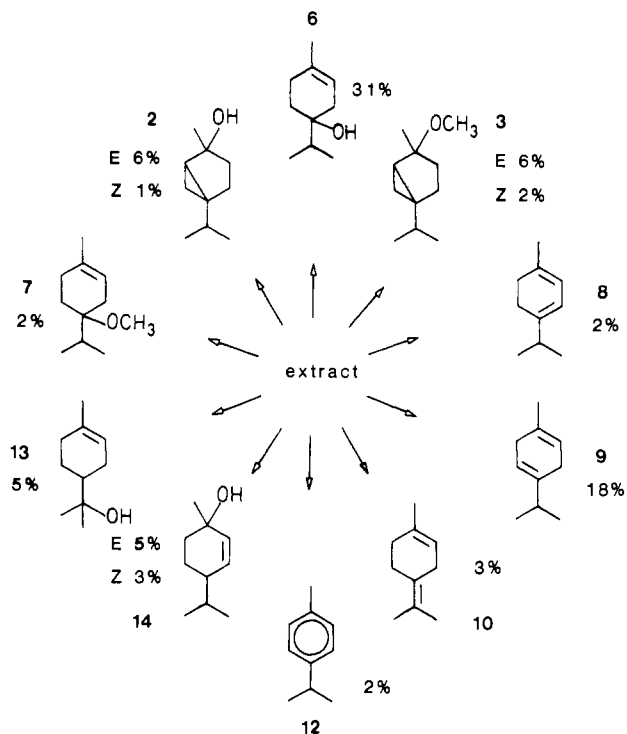


Figure 3. Distribution of monoterpenes obtained by methanolysis of the acetone extract (B): 1-13, see Figures 1 and 2; 14, (*E*/*Z*)-*p*-menth-2-en-1-ol.

RP-18 isolate from marjoram (Nitz et al., 1985). The ethers represented those of (*Z*)- and (*E*)-sabinene hydrate (3) and terpinen-4-ol (7). A considerable percentage of rearrangement occurred during treatment with methanol, especially to 6 and 9 (see Figure 3 and chromatogram in Figure 4).

The formation of ethyl esters of monoterpenes in alcoholic distillates of marjoram has already been described by Taskinen (1974). He also detected the ethers of the sabinene hydrates and terpinen-4-ol, besides a few minor ethers, when using ethanol for the distillative separation of flavor compounds.

In all these reactions the pattern of monoterpenes varies with the conditions applied for their isolation. The differing proportions of individual terpenes suggest their emergence from a common precursor, which rearranges to the observed compounds. In our previous work we as-

Table I. Relative Percentages of Monoterpenes Obtained from Marjoram Extracts or Leaf Material after Different Workup and Solvent Extraction

compound ^b	acetone	ethanol		NH ₄ HCO ₃	ground leaf
	extract: water	extract ^a acid	MeOH	extract: NaBH ₄	
sabinene	1	+ ^c	+	3	9
α -terpinene	3	8	2	+	+
<i>p</i> -cymene	1	5	2	+	+
γ -terpinene	9	21	18	+	+
(<i>E</i>)-sabinene hydrate	8	3	6	4	5
(<i>E</i>)-sabinene hydrate methyl ether	-	-	6	-	-
(<i>Z</i>)-sabinene hydrate	37	5	2	58	35
(<i>Z</i>)-sabinene hydrate methyl ether	-	-	2	-	-
(<i>E</i>)- <i>p</i> -menth-2-enol	+	1	5	+	-
(<i>Z</i>)- <i>p</i> -menth-2-enol	+	1	3	+	-
terpinen-4-ol	28	52	31	6	+
terpinen-4-ol methyl ether	-	-	2	-	-
(<i>Z</i>)-sabinene hydrate acetate	-	-	-	11	32

^a See the Experimental Section. ^b Listed in order of elution from OV-1 and SE-54 columns. ^c Key: +, below 1%; -, not detectable.

sumed that the spontaneous hydrolysis and formation of methyl ethers could be explained with known reactions of terpene pyrophosphates (Bunton et al., 1979; Haley et al., 1969; Herriott, 1975; Astin and Whiting, 1975; Brems and Rilling, 1977; Cori et al., 1986). In order to get more information about the terpene moiety leading to the rearranged monoterpenes, reductive cleavage of this precursor was performed using sodium borohydride as reagent. The substrate was an extract prepared under conditions that should be suited to stabilize terpenoid pyrophosphates (Cramer and Rittersdorf, 1967; Cori and Rojas, 1985). (See the Experimental Section.)

The borohydride cleavage of a part of this extract (C) yielded 58% (*Z*)-sabinene hydrate, 11% of the acetate, and 4% of the (*E*)-alcohol. Additionally, smaller amounts of rearranged monoterpenes were present [terpinen-4-ol (6%), total terpene hydrocarbons (8%)]; this result suggested that the terpene moiety of the assumed precursor should possess sabinene hydrate structure.

An almost identical distribution was observed after similar treatment of a sample of the ethanol extract (A). The results obtained from the different workup procedures mentioned above are summarized in Table I.

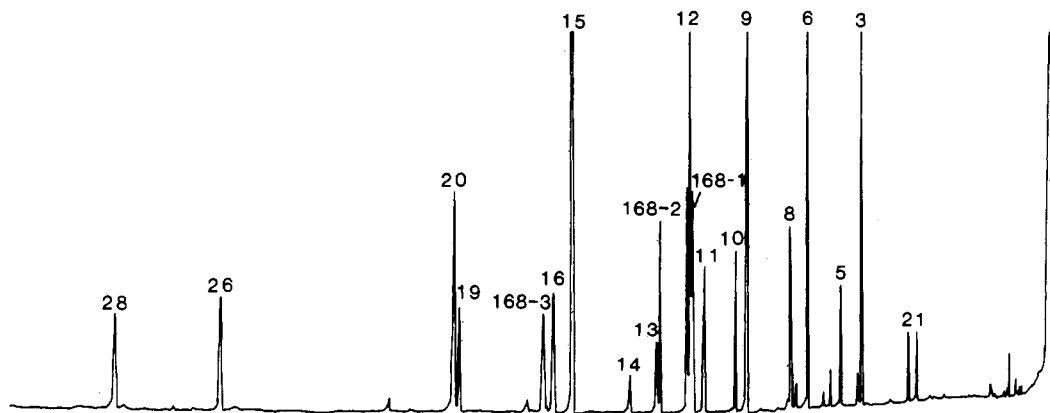


Figure 4. Chromatogram of the acetone extract (B) from marjoram after refluxing with methanol. Conditions: 1 μ L split; column OV-1. Peak numbers: 1, α -thujene; 2, α -pinene; 3, sabinene (1); 5, myrcene; 6, α -terpinene (8); 8, limonene (11); 9, γ -terpinene (9); 10, (*E*)-sabinene hydrate ((*E*)-2); 11, terpinolene (10); 168-1, (*E*)-sabinene hydrate methyl ether ((*E*)-3); 12, (*Z*)-sabinene hydrate ((*Z*)-2) + linalool (trace); 168-2, (*Z*)-sabinene hydrate methyl ether ((*Z*)-3); 13, (*E*)-*p*-menth-2-en-1-ol ((*E*)-14); 14, (*Z*)-*p*-menth-2-en-1-ol ((*Z*)-14); 15, terpinen-4-ol (6); 16, α -terpineol (13); 168-3, terpinen-4-ol methyl ether (7); 19, linalyl acetate; 20, (*Z*)-sabinene hydrate acetate (4); 26, caryophyllene; 28, bicyclogermacrene (tentatively identified).

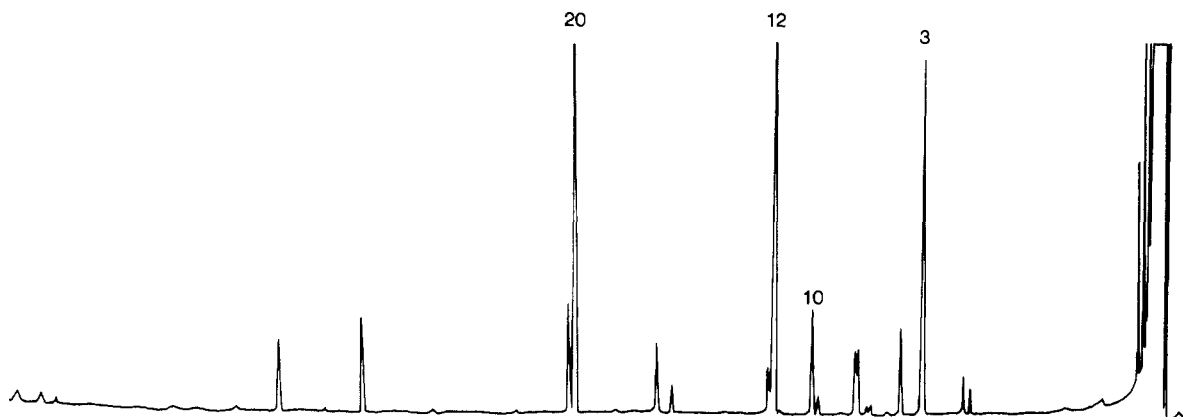


Figure 5. Chromatogram of pentane extract of marjoram after grinding with Na_2CO_3 . Conditions: $1 \mu\text{L}$ splitless; column OV-1. Peak numbers: see Figure 4.

Table II. Percentage of Main Components in Oil Isolated from Oil Glands (Fischer et al., 1987)^a

compound	% ^b
sabinene	10
(<i>E</i>)-sabinene hydrate	2
(<i>Z</i>)-sabinene hydrate	40–50
(<i>Z</i>)-sabinene hydrate acetate	20–30

^aDirect injections of glass capillaries filled with submicroliter amounts of essential oil of a few oil glands. ^bRange of values of a few repeated injections.

If the postulated reactive precursor should be a terpenoid pyrophosphate, the detection of the inorganic part must be possible; so the residue of the borohydride reaction, after extraction of the terpenes, was semiquantitatively analyzed for phosphate content. After precipitation of the phosphate as a barium salt and workup (see the Experimental Section), it was determined by a colorimetric quick-test set; calculated as pyrophosphate, it was approximately $0.6 \mu\text{mol}$ in the sample, compared to a content of the sabinene hydrates of $0.8 \mu\text{mol}$ in the organic extract. Thus, a possible sabinene hydrate–pyrophosphate precursor (5) seems to be conceivable with regard to stoichiometry and consistent with the known steps of terpene biosynthesis (Cane, 1986).

Attempts to synthesize 5 following known methods for the preparation of terpenoid pyrophosphates (Cori and Rojas, 1985; Davison et al., 1985; Cramer and Rittersdorf, 1967) were not successful, probably because of diverging properties of the sabinene hydrate skeleton: on the one hand, poor reactivity as a tertiary alcohol in nucleophilic substitution reactions and, on the other hand, the strong tendency to form rearranged products. So definite proof for the presence of this possible precursor form in marjoram could not be furnished.

Trituration Experiments. In order to obtain flavor extracts from marjoram without risking the observed chemical changes, triturations of the plant material under liquid nitrogen were prepared, followed by pentane extraction of these powders. Surprisingly, in the chromatograms of these extracts only three major peaks could be found (see Figure 5), representing sabinene (1), (*Z*)-sabinene hydrate (2), and its acetate (4), with percentages of 9%, 35%, and 32%, respectively. Addition of all compounds possessing the sabinene or sabinene hydrate skeleton gave up to 90% of the total monoterpene content. The dominance of these compounds in the oil glands of the marjoram leaf was confirmed by the analysis of oil that was isolated by means of fine-glass capillaries from the secretory cells and injected directly into the GC-MS

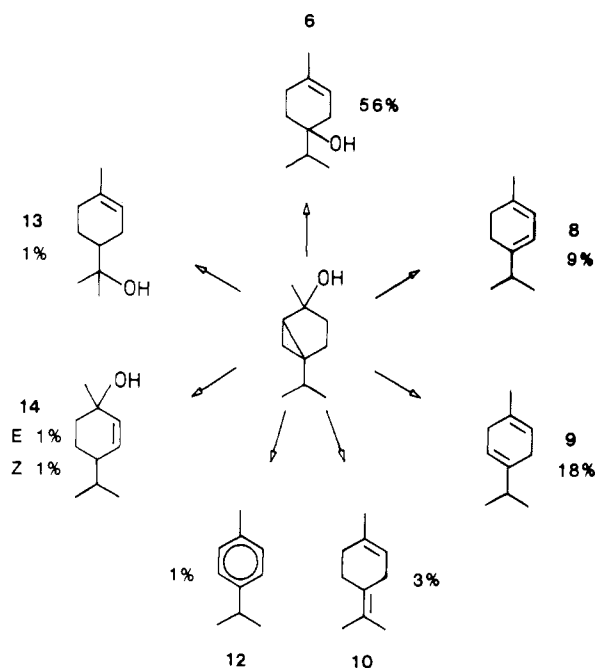


Figure 6. Distribution of monoterpenes obtained after acid-catalyzed rearrangement of (*E/Z*)-sabinene hydrate. Compound numbers: see Figures 1–3.

(Fischer et al., 1987) (see Table II).

Model Reactions. The dominance of terpenes with sabinene hydrate skeleton in the trituration and direct-injection experiments prompted us to study the rearrangement reactions of 2 and 4, the latter of which, though being a main component in extracts prepared under mild conditions, was never observed in considerable amounts in the distillates or essential oils. So the two stereoisomer sabinene hydrates 2 and their acetates 4 were synthesized, following the oxymercuration–demercuration procedure described by Brown and Geoghegan (1967) (see the Experimental Section).

A part of the synthesized mixture of (*E/Z*)-2 was taken into dilute sulfuric acid (pH 2), overlaid with pentane, and allowed to stand at room temperature for 16 h; the resulting distribution of monoterpenes was determined after extraction with pentane (see Figure 6). In this experiment again terpinen-4-ol (6) was dominating (56%); a control experiment without addition of acid expectedly gave no rearrangement products. After 1 h of heating of another sample of 2 under reflux in water, partial rearrangement occurred, again mainly to 6, while 67% of the sabinene hydrates remained unchanged. These experi-

ments show that the rearrangement of the sabinene hydrates gives rise to the same components as observed after "spontaneous hydrolysis" of marjoram extracts or RP-18 isolate (Nitz et al., 1985); the conditions necessary to start these reactions however are much more drastic when the free alcohol is used (acid catalysis, heat), in comparison to the ease of reaction in the aforementioned plant extracts (spontaneous hydrolysis with water, no acid, no heat). Owing to the cyclopropane ring, the structure of 2 seems to be similarly reactive as that of allylic terpene alcohols when protonated (compare the rearrangement reactions of linalool or nerol; see Engel and Tressl (1983) and Cori et al. (1986)); the configuration could be regarded as "pseudoallylic". The same statement also applies to sabinene, which under similar conditions also produces 6 and 8-10 (Cooper et al., 1973).

Rearrangement of (*Z*)-sabinene hydrate acetate (4) was at first studied in solvent extracts from marjoram that contained this ester; leaf material that had been stirred at room temperature in an aqueous buffer (pH 7.0) gave, after extraction with pentane, a distribution of monoterpenes very similar to that obtained by trituration (see above), with (*Z*)-sabinene hydrate 2 and the acetate 4 as main components. When leaf material was heated in the same buffer for 30 min, no more 4 was detectable, but appreciable amounts of the rearranged monoterpenes 6, 8, and 9 had been formed. Also, in the solvent extracts from ground leaf material the acetate vanished, when the extracts were heated in presence of water, and the rearrangement products occurred.

The percentage of (*Z*)-sabinene hydrate was approximately the same as in nonheated material; so it can be assumed that the observed rearranged components were formed at the expense of the acetate by application of heat, especially since the alcohol (*Z*)-sabinene hydrate had proven to be quite stable against heat, as mentioned above.

Similar rearrangement reactions were reported for linalyl acetate (Morin and Richard, 1984; Kubeczka and Schmaus, 1985), with the reservation that, in that case, the acetate proved to be partially stable at pH 7 (82% remained unchanged after 0.5-h reflux in water).

The instability of 4 against heat explains the observation that the acetone extract (B) did not contain the ester after spontaneous hydrolysis (see above), because this sample had been distilled in an SDE apparatus (Schultz et al., 1978) to remove the green pigments from the pentane extract, in contrast to the hydrolysate of the ethanol extract (A). Why 11% sabinene hydrate acetate is present at all in the ethanol extract—despite pretreatment with pentane to remove free flavor compounds—cannot be decided yet.

The apparent lability of 4 raised the question whether this compound would also produce the previously mentioned methyl ethers when treated with methanol.

So a synthesized sample of the (*Z*)-acetate was refluxed with methanol for 30 min, and the rearrangement products were determined after extraction with pentane. Indeed, the same methyl ethers were formed (ethers 3 and 7) as in equally treated RP-18 isolate or in marjoram extracts. Main component was unreacted acetate 4 (50%), besides the rearranged terpenes (6, 12%; 8 and 9, 4 and 7%). The portion of methyl ethers was for 3 8% *E* and 6% *Z* and for 7 3%.

In contrast to the proposal of Taskinen (1976), who explained the formation of, in his work, ethyl ethers of the sabinene hydrates and terpinen-4-ol with acid-catalyzed reactions of the free terpene alcohol, this formation of ethers seems to be due to reactions of sabinene hydrate

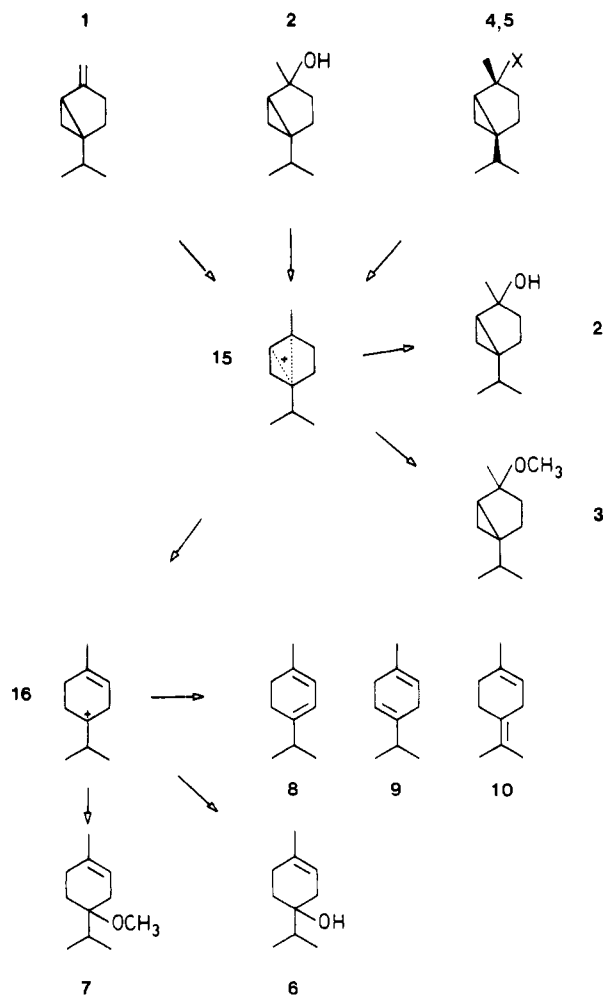


Figure 7. Proposed scheme for the reaction sequences leading to the pattern of rearranged monoterpenes in marjoram. Compound numbers: 1-10, see Figure 1; 16, terpinen-4-yl cation.

acetate (4) (as a free flavor compound) or of the postulated reactive precursor, possibly sabinene hydrate pyrophosphate (5) (bound form); both compounds facilitate addition and rearrangement reactions by an activated carbon-oxygen bond.

The initial step in this reaction sequence seems to be the formation of an intermediate carbocation with a structure derived from sabinene, as shown in Figure 7 (15). The depicted structure is that proposed by Cooper et al. (1973). The conditions necessary for the formation of this cation depend on the nature of the leaving group in the molecule (and, of course, on the chemical surrounding, i.e. solvent). When free sabinene hydrate, sabinene hydrate acetate, and the proposed activated precursor sabinene hydrate pyrophosphate are compared, the pyrophosphate group is by far the best leaving group, facilitating hydrolysis and rearrangement, followed by the acetate, which tends to rearrange with elevated temperature or with decreasing pH; the most stable structure in the series is that of sabinene hydrate, with the water molecule as leaving group. It is known from substitution or elimination reactions that the OH group needs to be protonated in order to be cleaved.

Beginning with this cation, a number of rearrangement, hydration (or alcohol addition), and deprotonation reactions occur, which lead to the well-known pattern of monoterpenes described in marjoram essential oils.

This proposed reaction scheme seems to be an inversion of the widely accepted "biogenetic isoprene rule" of Ruzicka (1953).

In the case of marjoram we have not been able to draw a distinct borderline between the two activated compounds responsible for the rearrangement reactions, sabinene hydrate acetate (main component in the oil glands besides sabinene hydrate) and the activated precursor form, presumably the sabinene hydrate pyrophosphate. It was also impossible to determine to what extent each individual component is involved in the formation of the rearranged products. If a little speculation is allowed concerning the biosynthesis of monoterpenes and the composition of essential oils, it could be stated that the biosynthetic capacity of marjoram exists in building up the sabinene skeleton, following the well-known enzymatically controlled sequences (see e.g. Croteau (1986)).

The formation of almost ubiquitous monoterpenes like 8-12, 6, and so on should then take place on the catabolic side, without enzyme control, but governed merely by "chemical" rules; thus, the reaction proceeds with formation of the thermodynamically favored conformations (tertiary terpinen-4-yl cation (16)), giving rise to rearranged monoterpene hydrocarbons by deprotonation, to alcohols by addition of water, and to ethers by addition of nucleophilic (short-chain) alcohols like methanol or ethanol.

As a conclusion it remains to be stressed that the analysis of essential oils is, at least in the case of marjoram, an analysis of artifacts. The degree of artifact formation is determined by the conditions applied to isolate the flavor compounds, and the resulting pattern of monoterpenes is not representative for the original composition of flavor compounds in the intact plant.

Essential oils from a lot of other plants also give hints for rearrangement reactions in the terpene patterns during workup, so that it can be presumed that labile structures like terpenoid esters or bound forms of terpenes are much more widespread than reported until now. So in analysis of flavor compounds extreme carefulness in working up the samples is essential, as already mentioned by Ohloff et al. (1985).

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Registry No. 1, 3387-41-5; 2, 546-79-2; 3, 115562-89-5; 4, 87553-42-2; 5, 115562-90-8; 6, 562-74-3; 7, 94281-60-4; 8, 99-86-5; 9, 99-85-4; 10, 586-62-9; 11, 138-86-3; 12, 99-87-6; 13, 98-55-5; (E)-14, 29803-81-4; (Z)-14, 29803-82-5; α -thujene, 2867-05-2; α -pinene, 80-56-8; myrcene, 123-35-3; linalool, 78-70-6; linalyl acetate, 115-95-7; caryophyllene, 87-44-5; bicyclogermacrene, 24703-35-3.

LITERATURE CITED

- Astin, K. B.; Whiting, M. C. "Allylic Carbonium Ions. Part II. Solvolysis and Cyclization of some Monoterpene 2,4-Dinitrophenyl Ethers". *J. Chem. Soc., Perkin Trans. 2* **1975**, 1161-1165.
- Brems, D. N.; Rilling, H. C. "On the Mechanism of the Prenyltransferase Reaction. Metal Ion Dependent Solvolysis of an Allylic Pyrophosphate". *J. Am. Chem. Soc.* **1977**, *99*, 8351-8352.
- Brown, H. C.; Geoghegan, P. "The Oxymercuration-Demercuration of Representative Olefins. A Mild, Convenient Procedure for the Markovnikov Hydration of the Carbon-Carbon Double Bond". *J. Am. Chem. Soc.* **1967**, *89*, 1522-1524.
- Bunton, C. A.; Cori, O.; Hachey, D.; Leresche, J.-P. "Cyclization and Allylic Rearrangement in Solvolyses of Monoterpenoids". *J. Org. Chem.* **1979**, *44*, 3238-3244.
- Cane, D. E. "Isoprenoid Biosynthesis. Stereochemistry of Cyclization of Allylic Pyrophosphates". *Acc. Chem. Res.* **1985**, *18*, 220-226.
- Cooper, M. A.; Holden, C. M.; Loftus, P.; Whittaker, D. "The Acid Catalyzed Hydration of Sabinene and α -Thujene". *J. Chem. Soc., Perkin Trans. 2* **1973**, 665-667.
- Cori, O.; Rojas, M. C. "Carbocyclases from *Citrus limonum*". *Methods Enzymol.* **1985**, *110*, 406-417.
- Cori, O.; Chayet, L.; Perez, L. M.; Bunton, C. A.; Hachey, D. "Rearrangement of Linalool, Geraniol, and Nerol and Their Derivatives". *J. Org. Chem.* **1986**, *51*, 1310-1316.
- Cramer, F.; Rittersdorf, W. "Hydrolysis of Phosphates and Pyrophosphates of some Monoterpene Alcohols—Reactions for Biosynthesis of Monoterpenes". *Tetrahedron* **1967**, *23*, 3015-3022.
- Croteau, R. "Biosynthesis of Cyclic Monoterpenes". In *Biogenesis of Aromas*; Parliment, T. H., Croteau, R., Eds.; ACS Symposium Series 317; American Chemical Society: Washington, DC, 1986; p 134.
- Davisson, V. J.; Woodside, A. B.; Poulter, C. D. "Synthesis of Allylic and Homoallylic Isoprenoid Pyrophosphates". *Methods Enzymol.* **1985**, *110*, 130-144.
- Engel, K.-H.; Tressl, R. "Formation of Aroma Components from Nonvolatile Precursors in Passion Fruit". *J. Agric. Food Chem.* **1983**, *31*, 998-1002.
- Fischer, N.; Nitz, S.; Drawert, F. "Original Flavour Compounds and Essential Oil Composition of Marjoram (*Majorana hortensis* Moench)". *Flavour Fragrance J.* **1987**, *2*, 55-61.
- v. d. Gathen, H.; Hörster, H. "Contribution to the Biological Role of Monoterpene Glycosides". In *Vorkommen und Analytik Ätherischer Öle*; Kubeczka, K.-H., Ed.; Thieme Verlag: Stuttgart, 1979; p 206.
- Granger, R.; Passet, J.; Lamy, J. "Sur les Essences Dites de Marjolaine". *Riv. Ital. Essenze Prof.* **1975**, *57*, 446-454.
- Haley, R. C.; Miller, J. A.; Wood, H. C. S. "Phosphate Esters Part II. The Formation of Monoterpene Hydrocarbons from Geranyl and Neryl Diphenyl Phosphates". *J. Chem. Soc. C* **1969**, 264-269.
- Herriott, A. W. "Rearrangement of Allylic Phosphonates". *J. Org. Chem.* **1975**, *40*, 801-802.
- Hörster, H. "Monoterpene Glycosides, a Discussion about their Biological Role and Experiments in Synthesizing these Compounds". In *Vorkommen und Analytik Ätherischer Öle*; Kubeczka, K.-H., Ed.; Thieme Verlag: Stuttgart, 1979; p 34.
- Koedam, A. "The Influence of Hydrodistillation on the Composition of Coniferous Oils". In *Ätherische Öle, Analytik, Physiologie, Zusammensetzung*; Kubeczka, K.-H., Ed.; Thieme Verlag: Stuttgart, 1982; p 188.
- Koedam, A.; Looman, A. "Effect of pH during Distillation on the Composition of the Volatile Oil from *Juniperus sabina*". *Planta Med., Suppl.* **1980**, 22-28.
- Koedam, A.; Scheffer, J. J. C.; Baerheim Svendsen, A. "Comparison of Isolation Procedures for Essential Oils IV. Leyland Cypress". *Perfum. Flavor.* **1980**, *5*, 56-65.
- Lossner, G. "Constituents of *Majorana hortensis*. II Cis- and trans-Sabinene Hydrates as Constituents of the Essential Oil". *Pharmazie* **1967**, *22*, 51-54.
- Lossner, G. "Phytochemical Considerations on Marjoram". *Planta Med.* **1968**, *16*, 54-57.
- Morin, P.; Richard, H. "Thermal Degradation of Linalyl Acetate During Steam Distillation". In *Progress in Flavour Research*; Adda, J., Ed.; Elsevier: Amsterdam, 1985; p 563.
- Nitz, S.; Fischer, N.; Drawert, F. "Bound Flavour Compounds in Plants I. Commun.: Volatile Terpenoid Compounds from Bound Precursors in *Majorana hortensis* Moench". *Chem. Mikrobiol. Technol. Lebensm.* **1985**, *9*, 87-94.
- Nykänen, I. "High resolution Gas Chromatographic-Mass Spectrometric Determination of the Flavour Composition of Marjoram (*Origanum majorana* L.) Cultivated in Finland". *Z. Lebensm. Unters. Forsch.* **1986**, *183*, 172-176.
- Ohloff, G.; Flament, I.; Pickenhagen, W. "Flavour Chemistry". *Food Rev. Int.* **1985**, *1*, 99.
- Pollter, C. "Isolierung und Identifizierung von Reaktionsprodukten aus gebundenen Aromavorstufen in *Majorana hortensis* Moench". Diploma Thesis, Technical University Munich, 1986.
- Ruzicka, L. "Isoprene Rule and the Biogenesis of Terpenic Compounds". *Experientia* **1953**, *9*, 357-367.
- Schmaus, G.; Kubeczka, K.-H. "The Influence of Isolation Procedures on the Composition of Essential Oils Containing Linalool and Linalyl Acetate". In *Essential Oils and Aromatic Plants*; Baerheim Svendsen, A., Scheffer, J. J. C., Eds.; Martinus Nijhoff/Dr. W. Junk: Dordrecht, 1985; p 127.
- Schultz, T. H.; Flath, R. A.; Mon, T. R.; Egging, S. B.; Teranishi, R. "Isolation of Volatile Compounds from a Model System".

- J. Agric. Food Chem. 1977, 25, 446-449.
- Still, W. C.; Kahn, M.; Mitra, A. "Rapid Chromatographic Technique for Preparative Separations with Moderate Resolution". *J. Org. Chem.* 1978, 43, 2923-2925.
- Takeo, T. "Production of Linalool and Geraniol by Hydrolytic Breakdown of Bound Forms in Disrupted Tea Shoots". *Phytochemistry* 1981, 20, 2145-2147.
- Taskinen, J. "Composition of the Essential Oil of Sweet Marjoram Obtained by Distillation with Steam and by Extraction and Distillation with Alcohol-Water Mixture". *Acta Chem Scand., Ser. B* 1974, B28, 1121-1128.
- Taskinen, J. "The Acid Catalyzed Reaction of some Monoterpene Alcohols in Aqueous Ethanol". *Int. Flavours Food Addit.* 1976, 7, 235-236.
- Williams, P. J.; Strauss, C. R.; Wilson, B.; Massy-Westropp, R. A. "Use of C₁₈ Reversed-Phase Liquid Chromatography for the Isolation of Monoterpene Glycosides and Norisoprenoid Precursors from Grape Juice and Wine". *J. Chromatogr.* 1982, 235, 471-480.
- Williams, P. J.; Strauss, C. R.; Wilson, B.; Massy-Westropp, R. A. "Glycosides of 2-Phenylethanol and Benzyl Alcohol in *Vitis vinifera* Grapes". *Phytochemistry* 1983, 22, 2039-2041.
- Wilson, B.; Strauss, C. R.; Williams, P. J. "Changes in Free and Glycosidically Bound Monoterpenes in Developing Muscat Grapes". *J. Agric. Food Chem.* 1984, 32, 919-924.

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Investigation of Volatile Constituents in Nectarines. 2. Changes in Aroma Composition during Nectarine Maturation

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Changes in the distribution of volatile constituents during the maturation of nectarines (*Prunus persica* L., Batsch var. *nucipersica* Schneid) were investigated by means of capillary gas chromatography. C₆ aldehydes and alcohols are the major components isolated from "green" fruit; in the course of fruit maturation, the concentrations of these C₆ components decrease and lactones become prominent constituents. γ - and δ -lactones develop differently depending upon their chain lengths. The last period of nectarine maturation, from "shipping-ripe" to "tree-ripe" fruit, is characterized by a significant increase in the amounts of aroma-contributing lactones having longer aliphatic side chains.

In the first part of our investigation of volatile constituents in nectarines (*Prunus persica* L., Batsch var. *nucipersica* Schneid), we characterized aroma patterns in different cultivars (Engel et al., 1988). Qualitative and quantitative distributions of volatiles were determined. Saturated and unsaturated γ - and δ -lactones were shown to be major aroma-contributing constituents in "tree-ripe" fruits.

The formation of flavor and aroma compounds in fruits is a dynamic process. Concentrations of volatiles cannot be seen as "fixed values", but they are subject to many variations; the degree of maturity or ripeness of the fruit plays a major role. Do et al. (1969) and Bayonove (1973) reported changes in the composition of volatiles during maturation and ripening of peaches. The knowledge about this phenomenon in nectarines is limited to the findings of Lim and Romani (1964). They demonstrated by means of gas chromatography an increase in the concentration of several volatile fractions during ripening of nectarines, but they did not identify individual components.

The objective of this study was to correlate the stages of nectarine fruit maturity with analytically detectable differences in the qualitative and quantitative composition of volatiles.

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EXPERIMENTAL SECTION

Materials. A white-flesh (P 89-56) and two yellow-flesh (Flavortop, P 62-27) nectarine cultivars were obtained from orchards of the Horticultural Crops Research Laboratory, U.S. Department of Agriculture, Fresno, CA. Flavortop is a commercially grown cultivar; P 89-56 and P 62-27 are presently being tested by the USDA in Fresno, CA. The fruits were harvested at four different degrees of maturity, which were determined according to size, color, and firmness (Haller, 1952). The selected degrees were classified as "green", "green-red", "shipping-ripe", and "tree-ripe"; they are shown on a growth curve of nectarines in Figure 1. Green-red and shipping-ripe are equivalent to the terms "immature" and "firm-mature", respectively, as they were defined on the maturity scale for peaches by Haller (1952).

Isolation of Volatiles. At each stage of maturity volatiles were isolated from 500 g of homogenized fruit pulp by liquid-liquid extraction with ether following the previously described procedure (Engel et al., 1988). (S)-(+)-Octanol-2 was used as standard.

Capillary Gas Chromatography—Mass Spectrometry (GC-MS). For capillary GC and GC-MS investigations a DB-Wax column (J&W Scientific, Folsom, CA; 60 m \times 0.32 mm; 0.25- μ m film thickness) was used. Instruments and conditions were as described previously (Engel et al., 1988).

RESULTS AND DISCUSSION

The maturation of nectarines can be divided into several stages. Figure 1 presents a growth curve typical for the cultivar Flavortop, showing the increase in fruit diameter over the season. Rapid development in stage I (increase of the pericarp) is followed by a rest period in stage II (hardening of the stone and development of the embryo) before the fruit finally reaches its maximal size and ma-