JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Structural and Sensory Characterization of Compounds Contributing to the Bitter Off-Taste of Carrots (*Daucus carota* L.) and Carrot Puree

ANDREAS CZEPA† AND THOMAS HOFMANN*,‡

Deutsche Forschungsanstalt für Lebensmittelchemie, Lichtenbergstrasse 4, D-85748 Garching, Germany

Sequential application of solvent extraction, gel permeation chromatography, and HPLC in combination with taste dilution analyses revealed that not a sole compound but a multiplicity of bitter tastants contribute to the bitter off-taste of cold-stored carrots and commercial carrot puree, respectively. Among these bitter compounds, 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (6-methoxymellein), 5-hydroxy-7-methoxy-2-methylchromone (eugenin), 2,4,5-trimethoxybenzaldehyde (gazarin), (*Z*)-heptadeca-1,9-diene-4,6-diin-3,8-diol (falcarindiol), (*Z*)-heptadeca-1,9-diene-4,6-diin-3-ol (falcarinol), and (*Z*)-3-acetoxy-heptadeca-1,9-diene-4,6-diin-8-ol (falcarindiol 3-acetate) could be identified on the basis of MS as well as 1D- and 2D-NMR experiments. Due to the low concentrations of <0.1 mg/kg and the high taste thresholds found for eugenin and gazarin, these compounds could be unequivocally excluded as important contributors to the bitter taste of carrots. Calculation of bitter activity values as the ratio of their concentration to their bitter detection threshold clearly demonstrated that neither in fresh and stored carrots nor in commercial carrot puree did 6-methoxymellein contribute to the bitter off-taste. In contrast, the concentrations of falcarindiol in stored carrots and, even more pronounced, in carrot puree were found to be 9- and 13-fold above its low bitter detection concentration of 0.04 mmol/kg, thus demonstrating that this acetylenic diol significantly contributes to the bitter taste of the carrot products investigated.

KEYWORDS: Bitter taste; carrots; taste dilution analysis; 6-methoxymellein; eugenin; gazarin; falcarinol; falcarindiol; falcarindiol 3-acetate

INTRODUCTION

Although modern breeding techniques and cultivar selection have been helpful to improve desired sensory quality attributes, carrots are able to produce sporadic bitter off-taste when exposed to abiotic stress during harvesting, transportation, storage, and processing. In consequence, this sporadic bitter taste is often the reason for consumer rejection of carrot products such as carrot puree in the infant diet and is, therefore, a major problem for vegetable processors.

Nearly 50 years ago, sensory studies revealed that bitter taste development occurred, in particular, in the phloem tissue of carrots when stored in the cold (*1*). The observed correlation between the intensity of the absorption maximum at 265 nm of a petroleum ether extract and the bitter overall taste of the carrot tissue led to the conclusion that the substance causing this strong absorption might be an analytical indicator for bitter perception (*2*). Chemical degradation, derivatization, and IR spectroscopy led to its identification as 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin, more commonly known as 6-methoxymellein (*3*).

Studies on the influence of ethylene on bitter taste development revealed that some abnormal phenols such as 6-methoxymellein, 5,7-dihydroxy-2-methylchromone, and 5-hydroxy-7-methoxy-2-methylchromone (eugenin) were produced, which were not present in carrot tissue stored under an atmosphere of air (*4*). In addition to the increase of the concentration of phenolic compounds, ethylene was recently reported to promote the formation of γ-terpinene, α-pinene, limonene, and *p*-cymene and to convert sucrose to fructose and glucose (*5*). In a very recent investigation on the influence of temperature and plant density on sensory quality and volatiles of carrots, a high content of terpenes was suggested to correlate with high sensorial scores for bitterness (*6*).

In carrots of different cultivars and maturity, 6-methoxymellein and its corresponding glucoside were detected, but on the basis of sensory studies the content of 6-methoxymellein seemed not to have any significant influence on the overall bitter taste

* Address correspondence to this author at the Institut für Lebensmittelchemie, Universität Münster, Corrensstrasse 45, D-48149 Münster, Germany [telephone (49) 251-83-33-391; fax (49) 251-83-33-396; e-mail thomas.hofmann@uni-muenster.de].

† Deutsche Forschungsanstalt für Lebensmittelchemie, Lichtenbergstrasse 4, D-85748 Garching, Germany.

‡ Institut für Lebensmittelchemie, Universität Münster, Corrensstrasse 45, D-48149 Münster, Germany.

10.1021/jf034085+ CCC: $25.00
Published on Web 05/17/2003

(*7*). To the contrary, bitter taste was always detectable when the carrot tissue contained eugenin at elevated levels. In contradiction, other investigations did not confirm any correlation between increasing eugenin content and bitter taste in carrots (*8*).

About 10 years ago, the first sensory studies on purified 6-methoxymellein revealed a bitter taste threshold concentration of 100 mg/kg in strained carrots (*9*). The concentation of 6-methoxymellein in various carrot batches was, however, below this threshold, thus suggesting that the isocoumarin might have only little effect on carrot bitterness.

Recent studies aimed at investigating how the chemical and sensory properties of carrots are influenced by environmental stress factors (*10*). The authors found higher scores for bitter as well as sour taste in stressed carrots, but in contradiction to the results of earlier studies (*9*), they attributed this flavor change to high levels of soluble phenolics and to the presence of 6-methoxymellein. In addition, abiotic stress was observed to increase ethylene production in carrot tissue, thus inducing higher amounts of 6-methoxymellein and lower amounts of sugars when compared to nonstressed carrots (*11*). The authors found correlations between the production of ethylene and the content of 6-methoxymellein, but not between 6-methoxymellein content and bitterness. They suggested that the increase of bitter taste intensity might be due to additional, yet unknown, bitter-tasting compounds, the production of which might be ethylene dependent, such as eugenin. Although recently a correlation between bitter taste and water-soluble phenolics was reported, the structures of these compounds have yet not been identified (*12*).

Besides these investigations on bitter-tasting carrot tissue, the so-called gazarin was identified as a bitter principle of carrot seeds, the structure of which had been later proven as 2,4,5-trimethoxybenzaldehyde (*13*, *14*). The role of gazarin in the bitter off-taste of carrot tissue is, however, not yet known.

This literature survey shows that despite extensive studies, the data available are very contradictory and that for none of the compounds detected in carrots could a correlation be found between the sensory evaluation of the bitter taste and the results obtained by instrumental analysis. This implies that yet unknown components might play a key role in evoking the bitter taste of carrots and their products. One reason for that lack of information might be that most studies focused primarily on quantitatively predominating compounds, rather than selecting the target compounds to be identified with regard to taste activity.

To answer the question as to which nonvolatile, key taste compounds are responsible for the typical taste of processed foods, recently, we developed a novel bioassay, which is based on the determination of the relative taste threshold of compounds in serial dilutions of HPLC fractions (*15*−*17*). This so-called taste dilution analysis (TDA), which has been proven to be a powerful screening procedure for the identification of intense taste compounds in foods, was yet not applied to bitter-tasting carrots. The identification of bitter indicator compounds in carrots is, however, essential to clarify the reasons leading to off-taste development in carrots.

The objectives of the present investigation were, therefore, to reinvestigate the taste impact of bitter tastants reported in the literature and to identify additional compounds contributing to the bitter taste of carrots and carrot puree by application of the TDA and to evaluate their contribution on the basis of a dose/activity relationship.

EXPERIMENTAL PROCEDURES

Chemicals. The following compounds were obtained commercially: 7-methoxycoumarin, ricinoleic acid, 2,4,5-trimethoxybenzaldehyde (Aldrich, Steinheim, Germany); sodium sulfate, sodium hydrogen carbonate, sodium hydroxide, lithium aluminum hydride (Merck, Darmstadt, Germany). Solvents were of HPLC grade (Merck). Fresh carrots were obtained commercially from a local vegetable market and were analyzed the same day. Bitter-induced carrots were obtained by slightly scratching the skin of fresh carrots with a knife and storing the carrots for 5 days at 5 °C. Carrot puree exhibiting a significant bitter off-taste was supplied by the German food industry.

Synthesis of (*Z*)-9-Octadecene-1,12-diol. A solution of ricinoleic acid (25 mmol) in dried tetrahydrofuran (20 mL) was dropped into a suspension of lithium aluminum hydride (30 mmol) in dried tetrahydrofuran (20 mL) at room temperature. The mixture was then refluxed for 30 min, cooled in an ice bath, and then hydrolyzed by careful addition of an aqueous $NaHCO_3$ solution (10% in water, 5 mL). After addition of a NaOH solution (20% in water, 10 mL), the organic layer was separated, and the aqueous phase was extracted with diethyl ether (3 × 25 mL). The organic phases were combined, dried over Na_2SO_4, and freed from solvent, affording (*Z*)-9-octadecene-1,12-diol (20 mmol; 80% yield) as a colorless oil: HRGC/MS(EI), *m*/*z* 55 (100), 67 (56), 82 (55), 81 (48), 68 (48), 96 (37), 95 (34), 97 (34), 41 (31), 54 (31); HRGC/MS(CI, NH_3), *m*/*z* 302 (100), 284 (27), 303 (23), 285 (20), 267 (16), 300 (7), 286 (6); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.91 (3H, t, 6.9 Hz), 1.30 (18H, m), 1.47 (6H, m), 2.08 (2H, q, 6.9 Hz), 2.22 (2H, m), 3.43 (2H, t, 6.5), 3.53 (1H, m), 5.50 (1H, m), 5.57 (1H, m); ^{13}C NMR (360 MHz , $CDCl_3$) δ 14.4 (CH), 23.1 (CH_2), 26.2 (CH_2), 26.2 (CH_2), 27.8 (CH_2), 29.6 (CH_2), 29.8 (CH_2), 29.9 (CH_2), 29.9 (CH_2), 30.0 (CH_2), 32.3 (CH_2), 33.1 (CH_2), 36.0 (CH_2), 37.3 (CH_2), 62.6 (CH_2), 71.5 (CH), 126.2 (CH), 132.7 (CH).

Sensory Analyses. *Training of the Sensory Panel.* Assessors were trained to evaluate the taste of aqueous solutions (3 mL each) of the following standard taste compounds by using a triangle test as described in the literature (*18*): saccharose (50 mmol/L) for sweet taste; lactic acid (20 mmol/L) for sour taste; NaCl (12 mmol/L) for salty taste; caffeine (1 mmol/L) for bitter taste; sodium glutamate (8 mmol/L, pH 5.7) for umami taste; tannin (gallustannic acid; 0.05%) for astringency. Sensory analyses were performed in a sensory panel room at 22−25 °C in three different sessions.

Determination of Taste Thresholds. The taste thresholds were determined by a triangle test using tap water (pH 6.5) as the solvent. The samples (3 mL) were presented in serial 1:1 dilutions in order of ascending concentrations. At the start of each session and before each trial, the subject rinsed with distilled water and expectorated. The samples, blanks as well as taste solutions, were swirled in the mouth briefly and expectorated. After indicating which glass vial contained the tastant, the participant received another set of two blanks and one taste sample. To prevent excessive fatigue, tasting began at a concentration level two steps below the individual threshold concentration that had been determined in a preliminary sensory experiment. The threshold values evaluated in three different sessions by eight panelists each were averaged. The values between individuals and separate sessions differed not more than one dilution step; that is, of 0.5 mmol/L for the reference bitter compound caffeine represents a range from 0.25 to 1.0 mmol/L.

Intensity Ranking Test. The bitter taste of commercial carrot puree, hackled fresh, and bitter-induced carrots was evaluated by the trained sensory panel using a scale from 0 (no bitter taste detectable) to 3 (strong bitterness).

Sequential Solvent Extraction of Carrots and Carrot Puree. Carrots (1 kg) and carrot puree (1 kg), respectively, were minced in a blender upon cooling and then extracted with hexane (3 × 800 mL) at room temperature. After filtration, the organic layers were combined and freed from solvent in vacuo to give the hexane-extractable compounds (fraction I). The residual carrot material was then extracted with ethanol (3 × 800 mL) upon stirring for 20 min at room temperature. After filtration, the ethanolic filtrates were combined and solvent was removed in vacuo, yielding the ethanol-soluble compounds (fraction II). The nonsoluble residue was, finally, extracted with water (3 × 800 mL) upon stirring for 20 min at room temperature, yielding

Table 1. Yields, Bitter Taste Thresholds and Bitter Activity Values (BAV) of Fractions I–III Isolated from Fresh Carrots, Stored Carrots, and Commercial Carrot Puree

sample	fraction[a]	yield[b] (g/kg)	bitter threshold[c] (g/kg)	BAV[d]
fresh carrots	I	0.3	2.1	1
	II	80.7	180.1	4
	III	13.3	72.4	2
stored carrots	I	0.4	1.1	4
	II	60.1	70.9	8
	III	11.4	46.3	2
carrot puree	I	0.9	0.8	11
	II	9.2	4.5	20
	III	74.8	83.3	9

[a] Individual fractions were isolated from carrot material by sequential extraction with hexane (I), ethanol (II), and water (III). [b] Yields were determined by weight. [c] The bitter taste detection threshold was determined in water using a triangle test. [d] Bitter activity values were calculated from the ratio of the concentration of the individual fractions in water (100 mL) and their bitter detection thresholds.

an aqueous extract upon filtration and freeze-drying (fraction III). The nonsoluble carrot residue was shown to be tasteless and was therefore discarded. Trace amounts of solvents were removed from fractions I−III, and after freeze-drying twice, the yields of the individual fractions I−III were determined by weight (**Table 1**).

Gel Permeation Chromatography (GPC). Fractions I and II were dissolved in ethanol (10 mL), filtered, and then applied onto a water-cooled glass column (100 cm × 50 mm, XK 50/100, Amersham Pharmacia Biotech, Freiburg, Germany), filled with a slurry of Sephadex LH-20 (Amersham Pharmacia Biotech) in ethanol. Chromatography was performed with at a flow rate of 2 mL/min and was monitored by means of a UV−vis detector (UV-1575, Jasco, Grossumstadt, Germany) operating at 302 nm. The 10 subfractions I-I−X and II-I−X were isolated from solvent fractions I and II, respectively, and were collected by a fraction collector; the solvent was removed at 30 °C in vacuo (45 mbar) and then freeze-dried twice. The material of each subfraction was used for the taste dilution analysis.

HPLC Fractionation of GPC Fractions I-VI, I-VIII, and II-VI. Fractions I-VI, I-VIII, and II-VI were dissolved in methanol/water (70:30, v/v; 3 mL) and then applied onto a glass column (1 × 10 cm^3) filled with RP-18 material (LiChroprep 25−40 μm, Merck) to separate the taste-active materials from taste-inactive carotenoids by flushing with methanol (40 mL). The effluent was collected, the solvent was removed in vacuo, and the nonvolatile residue was dissolved in methanol/water (70:30, v/v; 1 mL). After membrane filtration, aliquots (200 μL) were fractionated by semipreparative HPLC on RP-18, ODS-Hypersil, 5 μm (ThermoHypersil, Kleinostheim, Germany), in a 250 × 10 mm i.d. semipreparative scale using the following methanol/water gradients (flow rate = 4.5 mL/min): for GPC fractions I-VI and II-VI, starting with a mixture (70:30, v/v) of methanol and water, the methanol content was increased to 100% within 50 min, and then held at 100% for 10 min; for GPC-fraction I-VIII, starting with a mixture (40:60, v/v) of methanol and water, the methanol content was increased to 60% within 10 min, then increased to 80% in 15 min, then increased to 100% within 0.05 min, and, finally, held at 100% for 10 min. The effluents of the peaks were collected and freed from the solvent in vacuo, and the residues of the fractions obtained were analyzed by the taste dilution analysis.

Taste Dilution Analysis (TDA). GPC fractions I−X isolated from solvent fraction I (**Figure 1**) or II, respectively, or the pooled HPLC fractions isolated from GPC fractions VI and VIII (**Figure 2**), respectively, were taken up in water (1−4 mL) and then diluted 1+1 with drinking water. The serial dilutions of each of these fractions were then presented to the sensory panel in order of ascending concentrations, and each dilution was evaluated in a triangle test. The dilution at which a taste difference between the diluted extract and two blanks (tap water) could just be detected was defined as taste dilution (TD) factor (*15*, *16*). The TD factors evaluated by four different assessors in three different sessions were averaged. The TD factors between individuals and separate sessions did not differ by more than one dilution step.

Isolation of 3-Methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (6-Methoxymellein, 1) from GPC Fraction H-VIII. GPC fraction I-VIII isolated from bitter-induced carrots was fractionated by preparative thin-layer chromatography (TLC) on silica gel 60 (0.5 mm; Merck) using toluene/methanol (50:1, v/v) as the mobile phase. The fluorescent band detected at R_f 0.46 was scraped off, suspended in a mixture (90:10, v/v; 30 mL) of diethyl ether and methanol, and dried over Na_2SO_4. After filtration, the solvent was removed in vacuo, affording 6-methoxymellein (**1**) as a white amorphous powder in a purity of >99%: HRGC/MS(EI), *m*/*z* 164 (100), 208 (98; $[M]^+$), 165 (66), 190 (51), 179 (29), 209 (26), 78 (16), 69 (15), 162 (15), 119 (13); ^{1}H NMR (360 MHz, $CDCl_3$; DQF-COSY; arbitrary numbering of the carbon atoms refers to structure **1** in **Figure 3**) δ 1.51 [3H, d, 6.6 Hz, H−C(10)], 2.87 [2H, d, 6.6 Hz, H−C(8)], 3.85 [3H, s, H−C(11)], 4.67 [1H, m, H−C(9)], 6.25 [1H, d, 2.2 Hz, H−C(6)], 6.37 [1H, d, 2.7 Hz, H−C(4)], 11.25 [1H, s, H−C(3)]; ^{13}C NMR (360 MHz in $CDCl_3$; DEPT-135, HMQC, HMBC; arbitrary numbering of the carbon atoms refers to structure **1** in **Figure 3**) δ 20.6 [CH_3, C(10)], 34.8 [CH_2, C(8)], 55.5 [CH_3, C(11)], 75.4 [CH, C(9)], 99.4 [CH, C(6)], 101.5 [CH, C(4)], 106.1 [C, C(2)], 140.8 [C, C(7)], 164.5 [C, C(3)], 165.7 [C, C(5)], 169.8 [CO, C(1)].

Isolation of 5-Hydroxy-7-methoxy-2-methylchromone (Eugenin, 2) and 2,4,5-Trimethoxybenzaldehyde (Gazarin, 3) from GPC Fraction H-VIII. GPC fraction I-VIII isolated from bitter-induced carrots was fractionated by semipreparative HPLC on RP-18, ODS-Hypersil, 5 μm (ThermoHypersil), in a 250 × 10 mm i.d. semipreparative scale using the following solvent gradient (flow rate = 4.5 mL/min): starting with a mixture (40:60, v/v) of methanol and water, the methanol content was increased to 60% within 10 min, then increased to 80% in 15 min, then increased to 100% within 0.05 min, and finally, held at 100% for 10 min. The fractions eluting at 10−15 min (fraction I-VIII/3) and at 22−27 min (fraction I-−VI-II/6), respectively, were collected, the solvent was removed in vacuo, and the residue was freeze-dried. Fraction I-VIII/6 contained 5-hydroxy-7-methoxy-2-methylchromone (eugenin, **2**) as a white amorphous powder in a purity of >99%. In addition, comparison of retention times and GC-MS data with the synthetic reference compound led to the identification of trace amounts of 2,4,5-trimethoxybenzaldehyde (gazarin, **3**) in HPLC fraction I-VIII/3. Spectroscopic data of eugenin (**2**): HRGC/MS(EI), *m*/*z* 206 (100; $[M]^+$), 177 (96), 176 (50), 69 (43), 163 (40), 148 (39), 95 (38), 205 (36), 123 (32), 207 (26); ^{1}H NMR (400 MHz, $CDCl_3$; DQF-COSY; arbitrary numbering of the carbon atoms refers to structure **2** in **Figure 3**) δ 2.34 [3H, s, H−C(10)], 3.85 [3H, s, H−C(11)], 6.02 [1H, s, H−C(8)], 6.33 [1H, d, 2.2 Hz, H−C(4)], 6.36 [1H, d, 2.2 Hz, H−C(6)], 12.68 [1H, s, HO−C(3)]; ^{13}C NMR (360 MHz in $CDCl_3$; DEPT-135, HMQC, HMBC; arbitrary numbering of the carbon atoms refers to structure **2** in **Figure 3**) δ 22.7 [CH_3, C(10)], 55.7 [CH_3, C(11)], 92.5 [CH, C(6)], 97.9 [CH, C(4)], 105.3 [C, C(2))] 108.8 [CH, C(8)], 158.1 [C, C(9)], 162.3 [C, C(7)], 165.4 [C, C(3)], 166.8 [C, C(5)], 182.5 [C, C(1)]. Spectroscopic data of 2,4,5-trimethoxybenzaldehyde (**3**): HRGC/MS(EI), *m*/*z* 196 (100; $[M]^+$), 181 (72), 150 (67), 125 (65), 153 (58), 95 (56), 69 (49), 110 (48), 109 (47), 179 (45).

Quantification of 3-Methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (6-Methoxymellein, 1). Fresh carrots (100 g), which were stored at 3°C for up to 12 days (**Figure 4**), were minced while cooling, a methanolic solution of 7-methoxycoumarin (1.5 mg/2 mL) was added as the internal standard, and the mixture was homogenized. In addition, three batches of commercial carrot puree (100 g) showing pronounced bitter taste were spiked with 7-methoxycoumarin (1.5 mg). The carrot materials were then extracted with hexane (3 × 170 mL) upon stirring for 20 min at room temperature. The suspension was centrifuged (10 min, 4500 upm), and the combined organic layers were filtered and then freed from solvent in vacuo. The hexane-extractables were dissolved in ethanol (10 mL), membrane filtered, and separated by GPC on Sephadex LH-20 following the procedure described above. Fraction I-VIII was collected and analyzed by HPLC on RP-18, Nucleosil RP-18, 5 μm (Macherey-Nagel, Düren, Germany) in a 250 × 46 mm analytical scale, monitoring the effluent at 260 nm (flow rate = 0.8

mL/min). Starting with a mixture (40:60, v/v) of methanol and water, the methanol content was increased to 60%, kept for 15 min, and then increased to 80% within 15 min. Quantitation of 6-methoxymellein in carrot samples spiked with distinct amounts of purified 6-methoxymellein (0.5−30 mg/kg) revealed a recovery rate of 94% for the isocoumarin.

Isolation of (*Z*)-Heptadeca-1,9-diene-4,6-diin-3,8-diol (Falcarindiol, 4), (*Z*)-Heptadeca-1,9-diene-4,6-diin-3-ol (Falcarinol, 5), and (*Z*)-3-Acetoxyheptadeca-1,9-diene-4,6-diin-8-ol (Falcarindiol 3-Acetate, 6) from Carrot Puree. Following the procedure described above, GPC fractions I-VI (**Figure 5**) and II-VI (**Figure 6**) were isolated from commercial carrot puree (24 kg), and the bitter compounds were located by means of the TDA (**Figure 7**). For the isolation of taste compounds, GPC fraction II-VI was dissolved in *n*-pentane and applied onto the top of a water-cooled column (300 × 18 mm) filled with a slurry of silica gel (silica gel 60, Merck) in *n*-pentane. The chromatography was performed using the following sequence of solvent mixtures: *n*-pentane (fraction A; 110 mL), *n*-pentane/diethyl ether (fraction B; 90:10, v/v; 110 mL), *n*-pentane/diethyl ether (fraction C; 80:20, v/v; 110 mL), *n*-pentane/diethyl ether (fraction D; 70:30, v/v; 110 mL), *n*-pentane/diethyl ether (fraction E; 60:40, v/v; 110 mL), *n*-pentane/diethyl ether (fraction F; 50:50, v/v; 110 mL), followed by diethyl ether (110 mL). After removal of the solvent in vacuo, fractions D and E were dissolved in a mixture (70:30, v/v; 4 mL) of methanol and water, and, after membrane filtration, aliquots (200 μL) were fractionated by HPLC on RP-18, ODS-Hypersil, 5 μm, 120 A (ThermoHypersil), in a 250 × 10 mm semipreparative scale starting with a solvent mixture (70:30, v/v) of methanol and water and increasing the methanol content to 100% within 50 min, followed by an isocratic elution with methanol for 10 min (flow rate = 4.5 mL/min). The three peaks eluting after 17, 22.5, and 28 min were collected and, after removal of the solvent in vacuo and freeze-drying, (*Z*)-heptadeca-1,9-diene-4,6-diin-3,8-diol (falcarindiol, **4**), (*Z*)-heptadeca-1,9-diene-4,6-diin-3-ol (falcarinol, **5**), and (*Z*)-3-acetoxyheptadeca-1,9-diene-4,6-diin-8-ol (falcarindiol 3-acetate, **6**) were obtained as colorless oils in purities of >99%. Spectroscopic data of falcarindiol (**4** in **Figure 9**): GC-MS(EI), *m*/*z* 129 (100), 128 (83), 115 (78), 91 (77), 55 (73), 77 (59), 41 (56), 157 (46), 105 (45), 43 (45); GC-MS(CI, NH_3), *m*/*z* 260 (100), 154 (77), 225 (63), 242 (56), 243 (48), 204 (32), 172 (25), 261 (24), 244 (21), 205 (21); ^{1}H NMR (400 MHz, C_6D_6, DQF-COSY; arbitrary numbering of the carbon atoms refers to structure **4** in **Figure 9**) δ 0.91 [3H, t, 7.1 Hz, H−C(17)], 1.17 [8H, m, H−C(12−15)], 1.28 [2H, m, H−C(16)], 1.32 [1H, d, 6.3 Hz, HO−C(3)], 1.41 [1H, d, 4.5 Hz, HO−C(8)], 1.86 [2H, m, H−C(11)], 4.51 [1, dd, 7.6, 6.3 Hz, H−C(3)], 4.89 [1H, m, H_a−C(1)], 5.00 [1H, dd, 8.0, 4.5 Hz, H−C(8)], 5.23 [1H, m, H_b−C(1)], 5.33 [1H, m, 10.6, 7.5 Hz, H−C(10)], 5.48 [1H, m, 10.6, 8.0 Hz, H−C(9)], 5.87 [1H, ddd, 17.0, 10.2, 7.6 Hz, H−C(2)]; ^{13}C NMR (360 MHz in C_6D_6; DEPT-135, HMQC, HMBC; arbitrary numbering of the carbon atoms refers to structure **4** in **Figure 9**) δ 14.1 [CH, C(17)], 22.6 [CH_2, C(16)], 27.2 [CH_2, C(11)], 29.1 [CH_2, C(14)], 29.2 [CH_2, C(13)], 29.3 [CH_2, C(12)], 31.8 [CH_2, C(15)], 58.7 [CH, C(8)], 63.5 [CH, C(3)], 68.6 [C, C(6)], 70.3 [C, C(5)], 78.3 [C, C(4)], 79.9 [C, C(7)], 117.3 [CH_2, C(1)], 127.7 [CH, C(9)], 134.7 [CH, C(10)], 135.9 [CH, C(2)]. Spectroscopic data of falcarinol (**5** in **Figure 9**): GC-MS(EI), *m*/*z* 91 (100), 117 (86), 103 (81), 55 (75), 115 (70), 159 (67), 131 (53), 129 (48), 43 (47), 41 (41); GC-MS(CI, NH_3), *m*/*z* 244 (100), 245 (28), 246 (10), 247 (9), 227 (9), 262 (9), 229 (9), 260 (7), 206 (7), 243 (6); ^{1}H NMR (400 MHz, $CDCl_3$, DQF-COSY; arbitrary numbering of the carbon atoms refers to structure **5** in **Figure 9**) δ 0.88 [3H, t, 7.0 Hz H−C(17)], 1.28 [8H, m, H−C(13−16)], 1.35 [2H, m, H−C(12)], 1.85 [1H, d, 6.5 Hz, HO−C(3)], 2.02 [2H, m, H−C(11)], 3.03 [2H, d, 6.9 Hz, H−C(8)], 4.91 [1H, m, H−C(3)], 5.24 [1H, m, H_a−C(1)], 5.38 [1H, m, H−C(9)], 5.46 [1H, m, H_b−C(1)], 5.52 [1H, m, H−C(10)], 5.94 [1H, ddd, 5.3, 10.2, 17.0 Hz, H−C(2)]; ^{13}C NMR (360 MHz in $CDCl_3$; DEPT-135, HMQC, HMBC; arbitrary numbering of the carbon atoms refers to structure **5** in **Figure 9**) δ 14.1 [CH, C(17)], 17.7 [CH_2, C(8)], 22.7 [CH_2, C(16)], 27.2 [CH_2, C(11)], 29.2 [CH_2, C(14)], 29.2 [CH_2, C(13)], 29.3 [CH_2, C(12)], 31.8 [CH_2, C(15)], 63.6 [CH, C(3)], 64.0 [C, C(6)], 71.4 [C, C(5)], 74.3 [C, C(4)], 80.3 [C, C(7)], 117.0 [CH_2, C(1)], 121.9 [CH, C(9)], 133.1 [CH, C(10)], 136.2 [CH, C(2)]. Spectroscopic data of falcarindiol 3-acetate (**6** in **Figure 9**): GC-MS(EI), *m*/*z* 43 (100), 129 (57), 157 (55), 128 (54), 161 (51), 115 (50), 171 (44), 91 (41), 55 (39), 133 (37); GC-MS(CI, NH_3), *m*/*z* 285 (100), 320 (24), 286 (20), 243 (13), 302 (11), 244 (9), 242 (9), 260 (5), 321 (5), 245 (5); ^{1}H NMR (400 MHz, $CDCl_3$, DQF-COSY; arbitrary numbering of the carbon atoms refers to structure **6** in **Figure 9**) δ 0.87 [3H, t, 7.0 Hz, H−C(17)], 1.28 [8H, m, H−C(13−16)], 1.39 [2H, m, H−C(12)], 1.79 [1H, d, 5.3 Hz, HO−C(8)], 2.09 [3H, s, H−C(19)], 2.10 [2H, m, H−C(11)], 5.16 [1H, dd, 5.3, 8.2 Hz, H−C(8)], 5.34 [1H, m, H_a−C(1)], 5.52 [1H, m, H−C(9)], 5.53 [1H, m, H_b−C(1)], 5.61 [1H, m, H−C(10)], 5.87 [1H, ddd, 5.7, 10.0, 16.7 Hz, H−C(2)], 5.92 [1H, m, H−C(3)]; ^{13}C NMR (360 MHz in $CDCl_3$; DEPT-135, HMQC, HMBC; arbitrary numbering of the carbon atoms refers to structure **6** in **Figure 9**) δ 14.1 [CH, C(17)], 20.9 [CH, C(19)], 22.6 [CH_2, C(16)], 27.7 [CH_2, C(11)], 29.1 [CH_2, C(14)], 29.2 [CH_2, C(13)], 29.3 [CH_2, C(12)], 31.8 [CH_2, C(15)], 58.6 [CH, C(8)], 64.5 [CH, C(3)], 68.6 [C, C(6)], 70.9 [C, C(5)], 74.8 [C, C(4)], 80.1 [C, C(7)], 119.7 [CH_2, C(1)], 127.7 [CH, C(9)], 132.0 [CH, C(10)], 134.7 [CH, C(2)], 169.4 [C, C(18)].

Quantification of (*Z*)-Heptadeca-1,9-diene-4,6-diin-3,8-diol (Falcarindiol, 4). Fresh carrots (20 g) as well as carrots (20 g) of the same batch that had been stored at 3 °C for 5 days (**Figure 4**) were minced while cooling, a methanolic solution of (*Z*)-9-octadecene-1,12-diol (1.0 mg/1.0 mL) was added as the internal standard, and the mixture was homogenized. In addition, three batches of commercial carrot puree (20 g) showing pronounced bitter taste were spiked with (*Z*)-9-octadecene-1,12-diol (1.0 mg) and then intimately mixed with Na_2SO_4 (100 g). After the addition of ethyl acetate (100 mL), the carrot material was homogenized by means of an ultraturrax and was then extracted three times with ethyl acetate (100 mL each) for 5 min. The organic layers were combined, the solvent was removed in vacuo, and the residue was dissolved in *n*-pentane (1 mL). After centrifugation (3000 upm), the supernatant was applied onto the top of a water-cooled glass column (200 × 10 mm) filled with silica gel 60 (63−200 μm, Merck) conditioned with *n*-pentane. After the column had been flushed with *n*-pentane/diethyl ether (80:20, v/v; 50 mL), analyte and internal standard were eluted with *n*-pentane/diethyl ether (40:60, v/v; 50 mL). The latter fraction was freed from solvent in vacuo, taken up in methanol (1 mL), membrane filtered, 1+5 diluted with methanol, and then analyzed by HRGC-FID.

High-Resolution Gas Chromatography—Mass Spectrometry (HRGC-MS). HRGC was performed with a type 5890 series II gas chromatograph (Fisons Instruments, Mainz, Germany) using SE-54 (30 m × 0.32 mm fused silica capillary, DB-5, 0.25 mm; J&W Scientific, Fisons, Mainz, Germany) by on-column injection at 40°C. After 2 min, the temperature of the oven was raised at 10 °C/min to 260 °C and held for 15 min isothermally. The flow of the carrier gas, helium, was 1.8 mL/min. MS analysis was performed with an MAT 95 S (Finnigan, Bremen, Germany) in tandem with the HRGC. Mass chromatography in the electron-impact mode (MS/EI) was performed at 70 eV and in the chemical ionization mode (MS/CI) at 115 eV with ammonia as the reactant gas.

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (BIO-TEK Kontron Instruments, Eching, Germany) consisted of two pumps (type 522), a Rheodyne injector (250 μL loop), and a UV−vis detector (type 535).

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C, DEPT-135, COSY, HMQC, and HMBC experiments were performed on AMX-400 and AM-360 spectrometers (Bruker, Rheinstetten, Germany). Deuterobenzene and deuterochloroform were used as solvents, and tetramethylsilane was used as the internal standard.

RESULTS AND DISCUSSION

Preliminary sensory studies on the taste of carrots demonstrated that scratching the skin of the carrots with a knife induced the development of a bitter off-taste upon cold storage. Besides these bitter-induced carrots, commercial carrot purees, which returned from the markets to the food companies due to consumer complaints, exhibited the bitter off-taste also. To gain first insight into the hydrophobicity of the compounds imparting that bitter off-taste, fresh and bitter-induced carrots as well as

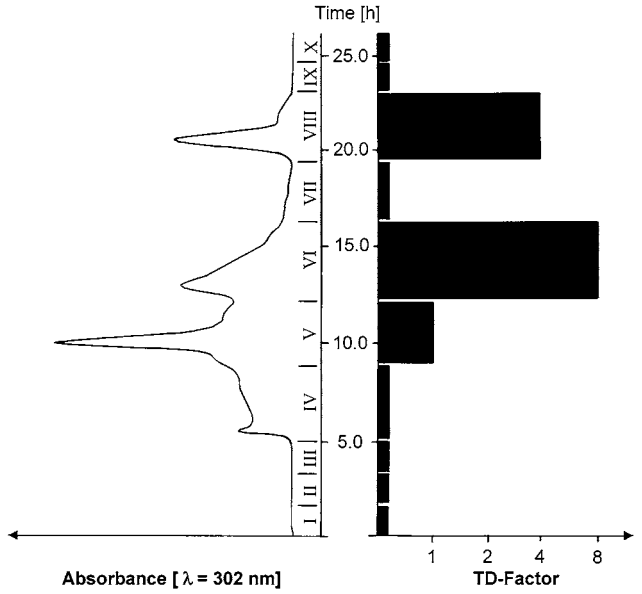

Figure 1. GPC chromatogram (left side) and taste dilution (TD) chromatogram (right side) of solvent fraction I isolated from bitter-induced carrots.

carrot puree were extracted sequentially with solvents of increasing polarity.

Bitter Activities of Solvent Extracts. After sequential extraction with hexane (fraction I), ethanol (fraction II), and water (fraction III), a nearly tasteless carrot material remained, indicating that the taste compounds had been fully isolated by the solvent extraction. After removal of the solvents from fractions I−III, the yield of each fraction was determined by weight (**Table 1**). Independent from the carrot material, the highest yields were obtained for the ethanolic solubles (fraction II), followed by the hexane and water extracts. To evaluate the bitter taste impact of these fractions, their taste detection thresholds have been determined using a triangle test, and bitter activity values (BAVs) were calculated from the quotient of the concentration and the threshold concentrations. The highest BAVs were found for fraction II; for example, the BAV of 20 was determined for the ethanolic fraction isolated from carrot puree, indicating that the concentration of that fraction in the carrot material is 20-fold above the bitter threshold (**Table 1**). In comparison, fraction II of the fresh carrots was evaluated with a 5-fold lower BAV of 4 only, which, however, increased to a value of 8 after cold storage. With the aim of identifying bitter taste compounds in carrot materials, fractions I and II isolated from the bitter-induced carrots as well as from the carrot puree were then screened for bitter compounds.

Sensory-Guided Fractionation of Bitter-Induced Carrots. To sort out the strongly taste-active compounds from the bulk of less taste-active or tasteless substances, first, the taste compounds in fraction I were separated from the carotenoids by means of GPC using Sephadex LH-20 as the stationary phase and ethanol as the mobile phase. The GPC chromatogram displayed in **Figure 1** was recorded by monitoring the effluent at 302 nm, and 10 fractions (fractions I−X) were collected separately. To evaluate their taste impact, these fractions were freeze-dried and then applied to the TDA. Due to their TD factors of 8 and 4, fractions I-VI and I-VIII were evaluated with the highest taste impacts, therefore, mainly contributing to the bitter taste of the hexane solubles of bitter-induced carrots (**Figure 1**, right side).

To have a more comprehensive picture on the compounds

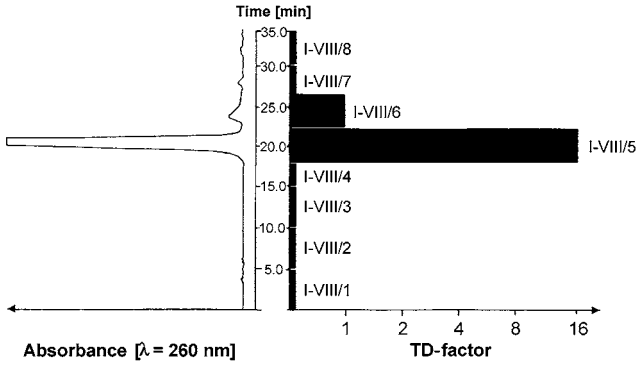

Figure 2. RP-HPLC chromatogram (left side) and taste dilution (TD) chromatogram (right side) of GPC fraction I-VIII isolated from bitter-induced carrots.

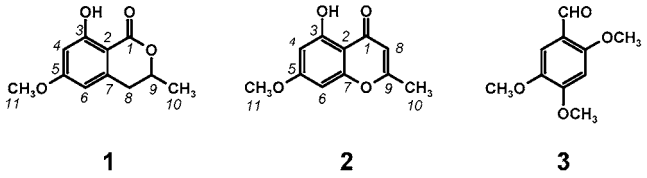

Figure 3. Structures of bitter-tasting 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin (6-methoxymellein, **1**), 5-hydroxy-7-methoxy-2-methylchromone (eugenin, **2**), and 2,4,5-trimethoxybenzaldehyde (gazarin, **3**), respectively, isolated from fractions I-VIII/5, I-VIII/6, and I-VIII/3 of bitter-induced carrots.

imparting the bitter taste of GPC fractions I-VI and I-VIII, these fractions were further separated by HPLC. Because GPC fraction VIII showed a very simple HPLC chromatogram, this fraction was analyzed first (**Figure 2**). Eight subfractions were collected and evaluated for their bitter taste impact by application of the TDA. Fraction I-VIII/5 was judged to have the highest TD factor of 16, followed by fraction I-VIII/6, showing bitter taste at the level of the detection threshold. The other six fractions did not show any taste impact. The compounds in fractions I-VIII/5 and I-VIII/6 were isolated by TLC and HPLC, freed from solvent in vacuo, and analyzed by HRGC-MS and ^{1}H and ^{13}C NMR spectroscopy.

HRGC-MS running in the CI mode revealed a molecular mass of 208 Da for the bitter compound isolated from fraction I-VIII/5. Signal integration in the ^{1}H NMR spectrum and the number of resonance signals in the ^{13}C NMR spectrum revealed the presence of 12 hydrogen atoms and 11 carbon atoms in the tastant, thus indicating a sum formula of $C_{11}H_{12}O_4$ for the bitter compound. Signal assignment by homo- and heteronuclear δ,δ-correlation experiments and comparison of the ^{1}H NMR data obtained with those reported in the literature (*4*) led to the unequivocal identification of the bitter tastant in fraction I-VIII/5 as 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin, known as 6-methoxymellein (**1** in **Figure 3**).

HRGC-MS analysis of the bitter compound present in fraction I-VIII/6 showed a molecular mass of 206 Da. ^{1}H and ^{13}C NMR as well as homo- and heteronuclear correlation experiments unequivocally identified the bitter compound as 5-hydroxy-7-methoxy-2-methylchromone, known as eugenin (**2** in **Figure 3**). The ^{1}H NMR spectroscopic data were identical with those reported earlier (*19*).

HRGC-MS analysis of the other HPLC fractions, which were evaluated with TD factors <1, led to the identification of 2,4,5-trimethoxybenzaldehyde (**3** in **Figure 3**), called gazarin, on comparison of the retention times and MS data with those

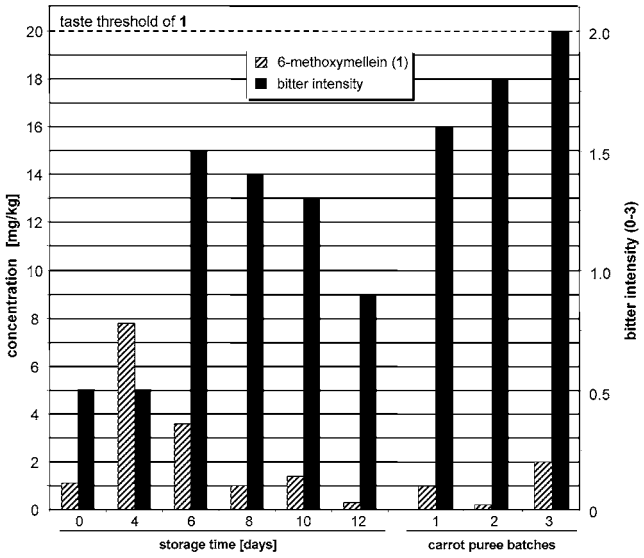

Figure 4. Concentration of 6-methoxymellein (**1**) and perceived bitter intensity in fresh and stored carrots as well as in commercial carrot purees.

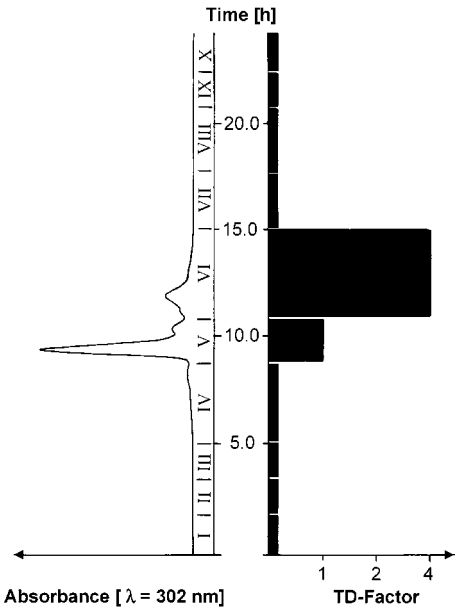

Figure 5. GPC chromatogram (left side) and taste dilution (TD) chromatogram (right side) of solvent fraction I isolated from carrot puree.

obtained from the commercially available reference compound. Although this compound was reported as a bitter compound in carrot seeds (*13*, *14*), the presence of gazarin in carrot tissue has yet not been reported.

Taste Impact of 6-Methoxymellein, Eugenin, and Gazarin. To evaluate the taste impact of 6-methoxymellein, eugenin, and gazarin, first, their bitter taste detection thresholds were determined in water by means of triangle tests. The lowest threshold concentration of 20 mg/kg was found for 6-methoxymellein. Comparison with literature data revealed that this threshold concentration in water is ∼5 times lower than the detection threshold of 6-methoxymellein in strained carrots; for example, 100 (*9*) and 94 mg/kg (*10*), respectively, have been reported. Compared to 6-methoxymellein, eugenin and gazarin showed somewhat higher threshold concentrations of 72 and 36 mg/kg in water, respectively.

To investigate the impact of these compounds in the bitter taste of carrot products, compounds **1**−**3** were quantified in fresh carrots, stored carrots, and commercial carrot puree by RP-HPLC, and the perceived bitter intensity of these samples was evaluated on a scale from 0 (not detectable) to 3 (intense bitterness). During the first 4 days of storage the carrots showed a low bitter intensity judged by a score of 0.5. After 6 days of storage, the bitter intensity increased and reached a maximum score of 1.5, thereafter decreasing slowly to reach a score of 0.8 after 12 days (**Figure 4**). Quantitative analysis of bitter compounds revealed that the fresh carrots contained 6-methoxymellein in concentrations of ∼1 mg/kg only. Storage of these carrots led to an increase of the amount of 6-methoxymellein running through a maximum of 7.7 mg/kg after 4 days, thereafter decreasing again and reaching a low level of <1 mg/kg after 12 days of cold storage. These data show that the formation of 6-methoxymellein is strongly dependent on the storage being well in accordance with earlier findings (*7*, *20*, *21*). Also, the commercial carrot purees contained the 6-methoxymellein in low concentrations only; for example, a maximum level of 2 mg/kg could be determined (**Figure 4**). On the contrary, these carrot puree samples showed comparatively high bitter intensity, which was scored between 1.5 and 2.0; for example, puree batch 2 was scored with the high bitter intensity of 1.8 but showed with 0.2 mg/kg the lowest concentration of 6-methoxymellein of all samples (**Figure 4**). Compared to 6-methoxymellein, eugenin and gazarine were present in the carrot samples in trace amounts of <0.1 mg/kg only (data not shown).

Relating the concentrations of compounds **1**−**3** with their bitter taste detection threshold demonstrated that the concentrations of 6-methoxymellein, eugenin, and gazarin in the carrots and carrot purees are far below their threshold concentrations; for example, the carrots stored for 6 days and carrot puree batch 3, respectively, showed high bitter intensities, but the concentrations of 6-methoxymellein were by factors of 6 and 10 below its detection threshold value. In contradiction to the results of some recent investigations (*4*, *10*, *12*), these studies clearly demonstrate that 6-methoxymellein, eugenin, and gazarin do not play any role in the bitter off-taste of carrots.

Taking all of these findings into consideration, it has to be concluded that other yet unknown compounds, that is, those eluting in GPC fraction VI, might be the key contributors to the bitter taste. Because, on the one hand, HPLC analysis of fractions I and II isolated from carrots and carrot puree demonstrated a very similar chemical composition, and, on the other hand, the carrot purees showed the highest scores in bitter intensity, the following fractionations were focused on the carrot puree.

Sensory-Guided Fractionation of Carrot Puree. The hexane solubles (fraction I; **Figure 5**) as well as the ethanol solubles (fraction II; **Figure 6**) isolated from carrot puree were separated by GPC, and the individual fractions were rated in bitter intensity by application of the TDA as described for the carrot tissue. Both fractions, I and II, isolated from the carrot puree showed the highest TD factors of 4 and 8, respectively, for GPC fraction VI. Comparing these results of the TDA with those obtained for the TDA of the hexane solubles isolated from the carrots (cf. **Figure 1**) demonstrated that fraction VIII containing the 6-methoxymellein was detected as a bitter fraction in carrots only, but not in the commercial purree. These data are well in line with the low concentration of 6-methoxymellein found in the puree (cf. **Figure 4**). In contrast, not only the fresh and stored carrots but also the carrot puree showed fraction VI as the most bitter fraction. Therefore, the following experiments were aimed at locating the bitter compounds in fraction VI of carrot puree by application of the HPLC/TDA approach. As an example,

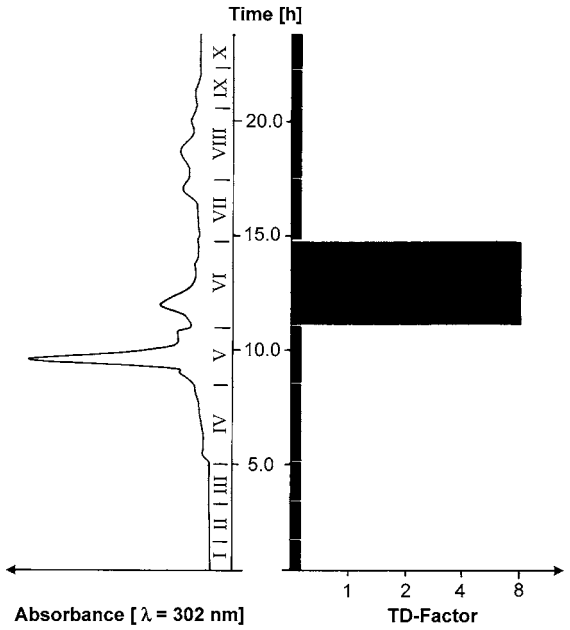

Figure 6. GPC chromatogram (left side) and taste dilution (TD) chromatogram (right side) of solvent fraction II isolated from carrot puree.

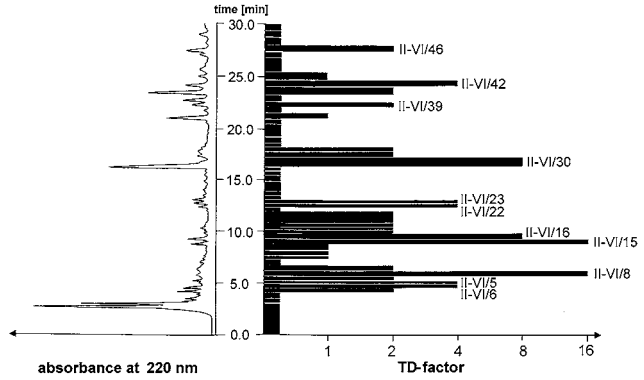

Figure 7. RP-HPLC chromatogram (left side) and taste dilution (TD) chromatogram (right side) of GPC fraction II-VI isolated from carrot puree.

the TDA chromatogram of the fraction II-VI isolated from carrot puree is given in **Figure 7**. Forty-nine fractions were collected, among which 26 fractions showed bitter taste with TD factors ranging between 1 and 16. This finding clearly showed that the bitter taste is not caused by a sole bitter tastant but by a multiplicity of individual taste compounds. The highest TD factors of 16 and 8 were found for fractions II-VI/8, II-VI/15, II-VI/16, and II-VI/30, respectively, thus contributing most to the bitter taste of GPC fraction II-VI. The TDA chromatograms (data not shown) obtained for fractions I−VI isolated from carrots and carrot puree were very similar to that given in **Figure 7**, indicating that the same multiplicity of bitter compounds were detectable in bitter-induced carrots.

Because a multiplicity of bitter tastants, each in low concentration, were present in carrot puree, we decided to focus our identification experiments on a bitter compound that can be used as a suitable indicator compound for measuring bitter taste. To achieve this, the ethanolic extract isolated from 24 kg of commercial carrot purree was separated by GPC, and the bitter compound detected in fraction II-VI/30 was isolated by preparative HPLC. A colorless oil was obtained, which was analyzed by HRGC-MS as well as 1D- and 2D-NMR spectroscopy. The molecular weight of 260 Da, which was determined by HRGC-MS in the chemical ionization mode using ammonia as reactant

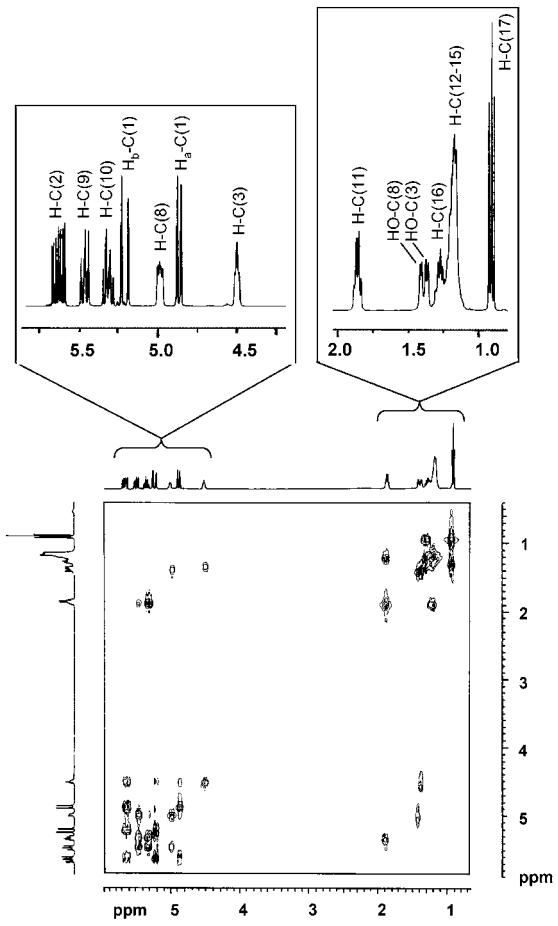

Figure 8. DQF-COSY spectrum and excerpts of the ^{1}H NMR spectrum (400 MHz, C_6D_6) of (*Z*)-heptadeca-1,9-diene-4,6-diin-3,8-diol (falcarindiol, **4**) isolated from fraction II-VI/30 of carrot puree.

gas, in combination with the 24 and 17 resonance signals detected in the ^{1}H and the ^{13}C NMR spectra, suggested a sum formula of $C_{17}H_{24}O_2$ for the bitter tastant.

Heteronuclear single-quantum correlation spectroscopy (HSQC) allowed the assignment of the signals at 0.91, 1.17, and 1.28 ppm, integrating for three, eight, and two protons as the methyl group H−C(17) and the methylene groups C(12)−C(15) and C(16), respectively. This was further strengthened by a homonuclear δ,δ-correlation experiment (DQF-COSY) demonstrating the strong coupling between these methylene protons and showing an additional coupling between the multiplet at 1.17 ppm and the multiplet of the methylene group H−C(11) resonating at 1.86 ppm (**Figure 8**). In addition, the COSY spectrum showed coupling of H−C(11) with the methine proton H−C(10) detected at 5.33 ppm and between the latter proton and the methine hydrogen H−C(9) resonating at 5.48 ppm. From the high coupling constant of 10.6 Hz, the *Z* configuration of the double bond C(9)═C(10) was deduced as proposed for structure **2** in **Figure 9**. Finally, a double doublet was detected for H−C(8) coupling to the multiplet at 5.48 ppm as well as the doublet at 1.41 ppm, which was assigned as the hydroxy proton HO−C(8). The disappearance of the signal at 1.41 ppm upon H/D exchange by adding trace amounts of D_2O confirmed the proposed hydroxy group in the compound. In addition, homonuclear coupling was observed in a COSY experiment between proton H−C(3) or HO−C(3) and the olefinic proton C(2) and heteronuclear multiple bond correlation (HMBC) to the carbons resonating at 117.3 and 135.9 ppm. Due to their strong homonuclear coupling, these were assigned as the

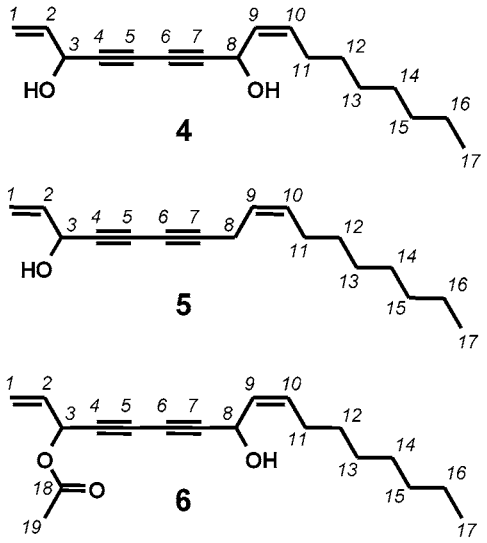

Figure 9. Structures of bitter-tasting (*Z*)-heptadeca-1,9-diene-4,6-diin-3,8-diol (falcarindiol, **4**), (*Z*)-heptadeca-1,9-diene-4,6-diin-3-ol (falcarinol, **5**), and (*Z*)-3-acetoxyheptadeca-1,9-diene-4,6-diin-8-ol (falcarindiol 3-acetate, **6**).

terminal methylene group H−C(1) and the methine group H−C(2). Unequivocal assignment of the quaternary carbon atoms resonating at 68.8, 70.3, 78.3, and 79.9 ppm could be successfully achieved by means of an HMBC experiment demonstrating a correlation between the protons H−C(8), HO−C(8), and H−C(9) and the carbon atom atoms resonating at 58.7, 79.9, and 68.8 ppm, respectively, which were assigned as the carbons of the triple bond C(8)≡C(7) and the acetylenic carbon C(6). Taking all of these spectroscopic data into consideration, the bitter compound in fraction II-VI/30 was unequivocally identified as (*Z*)-heptadeca-1,9-diene-4,6-diin-3,8-diol (**4** in **Figure 9**). Although this so-called falcarindiol was reported earlier to be present in Apiaceae, the sensory properties of that compound were as yet not known.

To investigate whether additional falcarindiol-type bitter compounds are present in GPC fraction II-VI, the bitter-tasting fractions detected by the TDA were screend by ^{1}H NMR spectroscopy for structure similarities. The bitter compounds in fractions II-VI/39 and II-VI/46 were found to have the aliphatic chain C(9)−C(17) as well as the conjugated triple bonds as shown for falcarindiol.

HRGC-MS analysis in the CI mode revealed a molecular weight of 244 for compound **5** isolated from fraction II-VI/39. Comparison with the GC-MS analysis of compound **4** demonstrated a mass difference of 16 amu, most likely corresponding to a lack of one oxygen atom in compound **5**. This was further confirmed by NMR spectroscopy enabling an unequivocal identification of the bitter tastant as (*Z*)-heptadeca-1,9-diene-4,6-diin-3-ol, the so-called falcarinol (**5** in **Figure 9**).

HRGC-MS analysis of compound **6** isolated from fraction II-VI/46 revealed a molecular weight of 302 Da, which is 42 amu above the molecular weight of compound **4**. On the basis of these findings the monoacetate of compound **4** was suggested as the bitter compound in fraction II-VI/46. 1D- and 2D-NMR experiments confirmed the structure of the tastant as (*Z*)-3-acetoxyheptadeca-1,9-diene-4,6-diin-8-ol, called falcarindiol 3-acetate (**6** in **Figure 9**).

To evaluate the sensory impact of these bisacetylenes, their bitter thresholds were determined in water by means of a triangle test and compared to the thresholds found for compounds **1**−**3** (**Table 2**). The bitter detection threshold of compound **4** was found to be 10 mg/kg (0.04 mmol/kg), which is 9-fold below the threshold concentration found for eugenin (**2**). A 2-fold higher threshold concentration of 20 mg/kg water (0.08 mmol/kg) was determined for falcarinol (**5**), whereas compound **6** was evaluated with a comparatively high bitter taste threshold of 60 mg/kg water (0.2 mmol/kg). Besides the bitter note, the acetate **6** exhibited a burning sensation on the tongue and the back of the throat at a lower threshold of 15 mg/kg.

Table 2. Bitter Taste Thresholds of Compounds Identified in Carrots and Carrot Puree

compound	threshold concentration[a] (mmol/kg)
6-methoxymellein (**1**)[b]	0.10
eugenin (**2**)[b]	0.35
gazarin (**3**)[b]	0.18
falcarindiol (**4**)[c]	0.04
falcarinol (**5**)[c]	0.08
falcarindiol 3-acetate (**6**)[c]	0.20

[a] The bitter taste detection threshold was determined in table water using a triangle test. [b] Structure of compound is given in **Figure 3**. [c] Structure of compound is given in **Figure 9**.

Table 3. Bitter Intensity and Concentration of 6-Methoxymellein (**1**) and Falcarindiol (**4**) in Commercial Carrot Puree and Fresh Carrots

sample[b]	bitter intensity[c]	concentration[a] (mg/kg) of **1**	concentration[a] (mg/kg) of **4**	BAV of **4**[d]
fresh carrots	0.5	2.0	41.0	4
fresh carrots	0.5	2.0	45.0	5
stored carrots	1.0	3.5	87.0	9
carrot puree batch 1	1.6	1.0	111.0	11
carrot puree batch 2	1.8	0.2	110.0	11
carrot puree batch 3	2.0	2.0	133.0	13

[a] The quantitative data represent the means of triplicates. [b] Fresh carrots were obtained from a local market; stored carrots were obtained by incubating the fresh carrots for 5 days at 3 °C; commercial carrot purees were those with consumer complaints due to significant bitter taste. [c] The bitter intensity was evaluated on a scale from 0 (not detectable) to 3 (strong bitter). [d] The bitter activity value was calculated from the ratio of the concentration and the detection threshold of falcarindiol (10 mg/kg).

Contribution of Falcarindiol (4) to the Bitterness of Carrots and Carrot Puree. To estimate the contribution of the most intensely bitter-tasting compound **4** to the overall bitter taste of carrot products, falcarindiol was quantified in fresh carrots, carrots stored for 5 days at 3 °C, and three carrot puree batches showing consumer complaints by HRGC-FID using (*Z*)-9-octadecene-1,12-diol as the internal standard. As given in **Table 3**, fresh carrots showing a bitter intensity of 0.5 contained falcarindiol in concentrations of 41 and 45 mg/kg, respectively. Calculation of the BAV revealed that the concentration of compound **4** was 4- or 5-fold above the threshold concentration. After the carrots had been stored for 5 days at 3 °C, bitter intensity showed an increased score of 1.0, and, in parallel, the amount of falcarindiol increased to 87 mg/kg, corresponding to a BAV of 9. The highest bitter scores and highest amounts of falcarindiol were determined in the carrot purees (**Table 3**). Among the carrot purees, batch 3 was judged to have the highest bitter intensity of 2.0 and contained 133 mg/kg falcarindiol, indicating that the concentration of that bitter tastant is 13-fold above its detection threshold. These data clearly demonstrate falcarindiol as a contributor to the bitter taste of carrots.

Comparative quantitative analysis of 6-methoxymellein revealed that the concentrations of the isocoumarin **1** are below 3.5 mg/kg in all samples analyzed and, therefore, are significantly below its bitter detection threshold of 20 mg/kg.

Although these bisacetylenes were already identified in various Apiaceae (*22*−*26*), their full spectroscopic data set and their sensory properties as well as their contribution to the bitter taste of carrots and carrot puree were yet not known in the literature. Knowledge of the structure, sensory activity, and concentrations of bitter indicator compounds such as falcarindiol in carrots and carrot puree is the basis to analytically objectify the bitterness of carrot products and might offer a new standard for an objective evaluation of the quality of carrot products. To objectify the role of carrot variety and growth and storage conditions in bitter taste development on a molecular level, the development of a versatile, straightforward analysis of these bitter compounds in carrot products is currently in progress and will be published soon.

ACKNOWLEDGMENT

We thank Martina Coklica and Kristin Kahlenberg for skillful assistance.

LITERATURE CITED

(1) Yamaguchi, H.; Howard, F. D.; McNelly, L. B. Observations on bitterness of California grown carrots. *Plant Dis. Rep.* **1955**, *39*, 302.

(2) Sondheimer, E.; Phillips, W. F.; Atkin, J. D. Bitter flavor in carrots. I. A tentative spectrophotometric method for the estimation of bitterness. *Food Res.* **1955**, *20*, 659−665.

(3) Sondheimer, E. The isolation and identification of 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin from carrots. *J. Am. Chem. Soc.* **1957**, *79*, 5036−5039.

(4) Coxon, D. T.; Curtis, R. F.; Price, K. R.; Levett, G. Abnormal metabolites produced by *Daucus carota* roots stored under conditions of stress. *Phytochemistry* **1973**, *12*, 1881−1885.

(5) Seljasen, R.; Hoftun, H.; Bengtsson, G. B. Sensory quality of ethylene-exposed carrots (*Daucus carota* L, cv "Yukon") related to the contents of 6-methoxymellein, terpenes and sugars. *J. Sci. Food Agric.* **2000**, *81*, 54−61.

(6) Rosenfeld, H. J.; Aaby, K.; Lea, P. Influence of temperature and plant density on sensory quality and volatile terpenoids of carrot (*Daucus carota* L.) root. *J. Sci. Food Agric.* **2002**, *82*, 1384−1390.

(7) Müller, H. Studies on the formation of 3-methyl-6-methoxy-3,4-dihydroisocumarin in carrots during storage (in German). *Phytopathol. Z.* **1978**, *93*, 241−248.

(8) Sarkar, S. K.; Phan, C. T. Naturally-occurring and ethylene-induced phenolic compounds in carrot root. *J. Food Prot.* **1979**, *42*, 526−534.

(9) Yoshino, N.; Kawaguchi, T.; Tokuoka, K.; Ishitani, T.; Hirata, T. 6-Methoxymellein levels in fresh carrots in relation to the sensory quality. *J. Jpn. Soc. Food Sci. Technol.* **1993**, *40*, 17−21.

(10) Talcott, S. T.; Howard, L. R. Chemical and sensory quality of processed carrot puree as influenced by stress-induced phenolic compounds. *J. Agric. Food Chem.* **1999**, *47*, 1362−1366.

(11) Seljasen, R.; Bengtsson, G. B.; Hoftun, H.; Vogt, G. Sensory and chemical changes in five varieties of carrot (*Daucus carota* L) in response to mechanical stress at harvest and post-harvest. *J. Sci. Food Agric.* **2001**, *81*, 436−447.

(12) Talcott, S. T.; Howard, L. R.; Brenes, C. H. Factors contributing to taste and quality of commercially processed strained carrots. *Food Res. Int.* **2001**, *34*, 31−38.

(13) Starkovsky, N. A. A new constituent of *Daucus carota* L.: 2,4,5-trimethoxy-benzaldehyde. *J. Org. Chem.* **1962**, *27*, 3733−3734.

(14) Khafagy, S. M.; Nazmi Sabri, N.; Abou Donia, A. H. Chemical characterization and preliminary pharmacological screening of the bitter principle isolated from *Daucus carota* L. var Boissieri Schweinf. Growing in Egypt. *J. Drug Res.* **1979**, *11*, 115−120.

(15) Frank, O.; Ottinger, H.; Hofmann, T. Characterization of an intense bitter-tasting 1*H*,4*H*-quinolizinium-7-olate by application of the taste dilution analysis, a novel bioassay for the screening and identification of taste-active compounds in foods. *J. Agric. Food Chem.* **2001**, *49*, 231−238.

(16) Ottinger, H.; Bareth, A.; Hofmann, T. Characterization of natural "cooling" compounds formed from glucose and L-proline in dark malt by application of taste dilution analysis. *J. Agric. Food Chem.* **2001**, *49*, 1336−1344.

(17) Frank, O.; Jezussek, M.; Hofmann, T. Sensory activity, chemical structure and synthesis of Maillard generated, bitter tasting 1-oxo-2,3-dihydro-1*H*-indolizinium-6-olates. *J. Agric. Food Chem.* **2003**, *51*, 2693−2699.

(18) Wieser, H.; Belitz, H.-D. Studies on the structure/activity relationship of bitter tasting amino acids and peptides (in German). *Z. Lebensm. Unters. Forsch.* **1975**, *159*, 65−72.

(19) Ali, A. A.; Makboul, M. A.; Attia, A. A.; Ali, D. T. Chromones and flavans from *Pancratium maritium*. *Phytochemistry* **1990**, *29*, 625−627.

(20) Kurosaki, F.; Nishi, A. Isolation and antimicrobial activity of the phytoalexin 6-methoxymellein from cultured carrot cells. *Phytochemistry* **1983**, *22*, 669−672.

(21) Marinelli, F.; Ronchi, V. N.; Salvadori, P. Elicitor induction of enzyme activities and 6-methoxymellein production in carrot cell suspension culture. *Phytochemistry* **1994**, *35*, 1457−1460

(22) Bohlmann, F.; Niedballa, U.; Rode, K.-M. On novel polyines with C_{17}-chain (in German). *Chem. Ber.* **1966**, *99*, 3552−3558.

(23) Bentley, R. K.; Bhattacharjee, D.; Jones, E. R. H.; Thaller, V. Natural acetylenes. Part XXVIII. C_{17}-polyacetylenic alcohols from the umbellifer *Daucus varota* L. (carrot): alkylation of benzene by acetylenyl(vinyl)carbinols in the presence of toluene-*p*-sulphonic acid. *J. Chem. Soc., Sect. C* **1969**, 685−688.

(24) Kemp, M. S. Falcarindiol: an antifungal polyacetylene from *Aegopodium podagraria*. *Phytochemistry* **1978**, *17*, 1002.

(25) Miyazawa, M.; Shimamura, H.; Bhuva, R. C.; Nakamura, S.; Kameoka, H. Antimutagenic activity of falcarindiol from *Peucedanum praeruptorum*. *J. Agric. Food Chem.* **1996**, *44*, 3444−3448.

(26) Eckenbach, U.; Lampman, R. L.; Seigler, D. S.; Ebinger, J.; Novak, R. J. Mosquitocidal activity of acetylenic compounds from *Cryptotaenia canadensis*. *J. Chem. Ecol.* **1999**, *25*, 1885−1893.

Received for review January 28, 2003. Revised manuscript received April 11, 2003. Accepted April 13, 2003. This research project was supported by the FEI (Forschungskreis der Ernährungsindustrie e.V., Bonn), the AiF and the Ministry of Economics and Labor. AiF-Project No.: 12226 N.

JF034085+