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# Characterization of the Key Aroma Compounds in Pink Guava (*Psidium guajava* L.) by Means of Aroma Re-engineering Experiments and Omission Tests

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Seventeen aroma-active volatiles, previously identified with high flavor dilution factors in fresh, pink Colombian guavas (*Psidium guajava* L.), were quantified by stable isotope dilution assays. On the basis of the quantitative data and odor thresholds in water, odor activity values (OAV; ratio of concentration to odor threshold) were calculated. High OAVs were determined for the green, grassy smelling (*Z*)-3-hexenal and the grapefruit-like smelling 3-sulfanyl-1-hexanol followed by 3-sulfanylhexyl acetate (black currant-like), hexanal (green, grassy), ethyl butanoate (fruity), acetaldehyde (fresh, pungent), *trans*-4,5-epoxy-(*E*)-2-decenal (metallic), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (caramel, sweet), cinnamyl alcohol (floral), methyl (2*S*,3*S*)-2-hydroxy-3-methylpentanoate (fruity), cinnamyl acetate (floral), methional (cooked potato-like), and 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (seasoning-like). Studies on the time course of odorant formation in guava puree or cubes, respectively, showed that (*Z*)-3-hexenal was hardly present in the intact fruits, but was formed very quickly during crushing. The aroma of fresh guava fruit cubes, which showed a very balanced aroma profile, was successfully mimicked in a reconstitute consisting of 13 odorants in their naturally occurring concentrations. Omission tests, in which single odorants were omitted from the entire aroma reconstitute, revealed (*Z*)-3-hexenal, 3-sulfanyl-1-hexanol, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 3-sulfanylhexyl acetate, hexanal, ethyl butanoate, cinnamyl acetate, and methional as the key aroma compounds of pink guavas.

**KEYWORDS: Pink guava; *Psidium guajava* L.; stable isotope dilution assay; aroma reconstitute; (*Z*)-3-hexenal formation; omission tests**

## INTRODUCTION

Guavas are the edible fruits of the guava tree *Psidium guajava* L., native to the American tropics. However, although their pleasant aroma, combining fruity, green, and tropical notes, is mainly responsible for their popularity, systematic studies on the key aroma compounds of guavas have been scarcely performed. To fill this gap the aroma-active compounds of Colombian pink guavas were recently screened by application of the aroma extract dilution analysis (AEDA) (*1*). High FD factors were found for 4-methoxy-2,5-dimethyl-3(2*H*)-furanone, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 3-sulfanylhexyl acetate, and 3-sulfanyl-1-hexanol followed by 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone, (*Z*)-3-hexenal, *trans*-4,5-epoxy-(*E*)-2-decenal, cinnamyl alcohol, ethyl butanoate, hexanal, methional, and cinnamyl acetate. Among them, six odorants, namely, 3-sulfanylhexyl acetate, 3-sulfanyl-1-hexanol, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone, *trans*-4,5-epoxy-(*E*)-2-decenal, and methional, were reported for the first time as guava constituents. The presence of 3-sulfanylhexyl acetate and 3-sulfanyl-1-hexanol in guavas was very recently confirmed by Clery and Hammond (*2*).

To prove and rank the aroma contribution of individual aroma compounds to the overall aroma of guavas, quantitative studies and aroma re-engineering experiments are a necessary further step. Thus, the aim of this study was to quantify the most odor-active compounds recently characterized by AEDA, to re-engineer the overall aroma based on the natural concentrations in guava, and finally to elucidate the key aroma compounds by means of omission tests.

## MATERIALS AND METHODS

**Fruits.** Pink-fleshed guavas (*P. guajava* L.), variety Regional Roja, were either from Puente Nacional (Santander, Colombia) and transported to Germany by air freight or of Colombian origin but purchased at a local market in Munich, Germany.

**Reference Odorants.** The following compounds were purchased from the commercial sources given in parentheses: **1** (Alfa Aesar,

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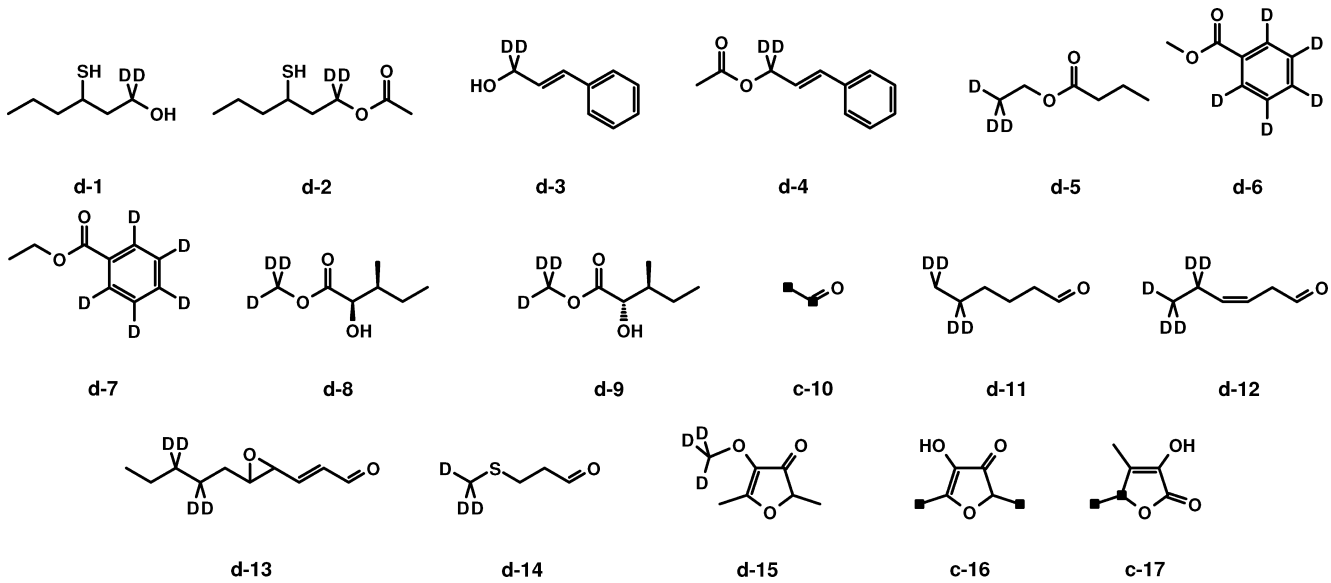


**Figure 1.** Structures of the deuterium- and $^{13}C$-labeled compounds used as internal standards in the stable isotope dilution assays: (■) positions of $^{13}C$ atoms.

Karlsruhe, Germany); **2**−**7**, **10**, **11**, **14**−**17** (Sigma-Aldrich Chemie, Taufkirchen, Germany). Compounds **2**, **8**, **9**, and **13** were synthesized according to previously published procedures: **2**, **8**, and **9** (*1*); **13** (*3*). (*Z*)-3-Hexenal (**12**) was synthesized by oxidation of (*Z*)-3-hexenol according to the procedure reported in ref *1* for the synthesis of the (*E*)-isomer.

**Isotopically Labeled Odorants (Figure 1).** The following compounds were synthesized according to the literature given in parentheses: **d-14** (*4*); **c-15** (*5*); **c-16** (*6*); **c-17** (*7*). Compounds **d-1**−**d-9** and **d-11**−**d-13** were synthesized as detailed in the following section. **C-10** was purchased from Aldrich.

**Syntheses.** *[1,1-$^2H_2$]-3-Sulfanyl-1-hexanol (**d-1**).* Following the reaction scheme used in ref *8* for the synthesis of [1,1-$^2H_2$]-3-sulfanyl-2-methyl-1-pentanol from 2-methyl-2-pentenal, [1,1-$^2H_2$]-3-sulfanyl-1-hexanol was prepared from (*E*)-2-hexenal as described below.

*(a) 3-(Acetylthio)hexanal.* In an argon atmosphere, thioacetic acid (27 mmol) was slowly added to a mixture of (*E*)-2-hexenal (20 mmol) and piperidine (0.25 mmol). After stirring for 18 h at ambient temperature, diethyl ether (20 mL) was added and the mixture was washed with hydrochloric acid (1 mol/L; 2 × 5 mL) followed by an aqueous saturated sodium hydrogen carbonate solution (10 mL). After drying over anhydrous sodium sulfate, the solvent was distilled off by means of a Vigreux column.

The intermediate showed the following mass spectrum (MS-EI): *m/z* (intensity in %) 44 (100), 40 (16), 45 (12), 43 (8), 41 (6), 39 (5), 43 (5), 42 (4), 36 (4), 73 (4).

*(b) 3-(Acetylthio)hexanoic Acid.* 3-(Acetylthio)hexanal and amidosulfonic acid (26 mmol) were dissolved in water/ethanol (2.5:1). Sodium chlorite (27 mmol) in ethanol/water (1:1; 30 mL) was slowly added, and the mixture was stirred for 5 h at ambient temperature. The mixture was extracted with diethyl ether (2 × 80 mL), and the combined etherial phases were filtered through a plug of silica gel and finally dried over anhydrous sodium sulfate. The solvent was removed by rotary evaporation.

The following mass spectrum (MS-EI) was obtained: *m/z* (intensity in %) 43 (100), 45 (47), 70 (27), 44 (23), 130 (23), 55 (22), 97 (21), 41 (19), 69 (19), 89 (19), 73 (15), 87 (15), 60 (10), 71 (10), 88 (10).

*(c) [1,1-$^2H_2$]-3-Sulfanyl-1-hexanol.* 3-(Acetylthio)hexanoic acid was dissolved in diethyl ether (5 mL) and dropwise added to a suspension of lithium aluminum deuteride (30 mmol) in diethyl ether (25 mL) under an argon atmosphere. After stirring of the refluxed mixture for 150 min, this was cooled to 0 °C, and saturated aqueous ammonium chloride solution (15 mL) was slowly added, followed by hydrochloric acid (2 mol/L; 10 mL). The organic phase was separated, washed with a saturated aqueous sodium hydrogen carbonate solution (10 mL), dried over anhydrous sodium sulfate, and made up to 100 mL. The concentration of the target compound was determined by GC (*9*): yield, 289 mg (11%); MS-EI, *m/z* (intensity in %) 55 (100), 41 (94), 61 (86), 58 (82), 56 (74), 57 (61), 102 (59), 69 (58), 85 (47), 42 (45), 101 (44), 83 (42), 47 (37), 43 (36), 39 (35), 68 (33), 84 (31), 89 (31), 75 (29), 59 (28), 45 (24), 136 (24); MS-CI (methanol), *m/z* (intensity in %) 119 (100; $M + H^+ - H_2O$).

*[1,1-$^2H_2$]-3-Sulfanylhexyl Acetate (**d-2**).* To a solution of [1,1-$^2H_2$]-3-sulfanyl-1-hexanol (**d-1**; 0.52 mmol) in dichloromethane (10 mL) was added dropwise acetyl chloride (1.31 mmol) in dichloromethane (3 mL) at 0 °C. After 2 h of stirring, the solvent and the excess acetyl chloride were removed by rotary evaporation.

The yield was determined by GC (*9*): 81 mg (87%); MS-EI, *m/z* (intensity in %) 43 (100), 90 (42), 118 (40), 85 (39), 75 (32), 55 (25), 56 (23), 89 (19), 57 (18), 69 (18), 41 (16), 87 (15), 84 (14), 103 (12), 42 (11), 45 (10); MS-CI (methanol), *m/z* (intensity in %) 119 (100; $M + H^+ - H_3C-COOH$).

*[1,1-$^2H_2$]-Cinnamyl Alcohol (**d-3**).* [1,1-$^2H_2$]-Cinnamyl alcohol was prepared by reduction of ethyl cinnamate with lithium aluminum deuteride following a procedure for the preparation of the unlabeled compound (*10*). In an argon atmosphere, ethyl cinnamate (10 mmol) was slowly added to lithium aluminum deuteride (5 mmol) at 0 °C with stirring. After 20 min, hydrochloric acid (2 mol/L; 50 mL) was added, followed by diethyl ether (50 mL). The organic phase was separated, washed with an aqueous saturated sodium hydrogen carbonate solution (10 mL), and dried over anhydrous sodium sulfate. The solvent was removed by means of a Vigreux column, and the crude product was purified by column chromatography to remove unreacted ethyl cinnamate. The solution (1 mL) was applied onto a water-cooled (12 °C) glass column (2 cm i.d.) filled with silica gel (25 g). The ester was removed by elution with pentane/diethyl ether (90:10; 150 mL), and [1,1-$^2H_2$]-cinnamyl alcohol was isolated with pentane/diethyl ether (50:50; 150 mL). The solution was made up to 200 mL, and the concentration of the target compound was determined by GC (*9*): yield, 1.11 g (82%); MS-EI, *m/z* (intensity in %) 93 (100), 136 (75), 92 (56), 78 (47), 91 (44), 79 (42), 117 (40), 106 (38), 77 (35), 94 (30), 107 (26), 51 (26), 80 (21), 116 (20), 135 (17), 118 (16), 104 (15), 119 (12), 63 (12), 103 (12), 39 (11), 105 (11); MS-CI (methanol), *m/z* (intensity in %) 119 (100; $M + H^+ - H_2O$), 120 (9).

*[1,1-$^2H_2$]-Cinnamyl Acetate (**d-4**).* Acetyl chloride (10 mmol) dissolved in dichloromethane (5 mL) was dropwise added to a solution of [1,1-$^2H_2$]-cinnamyl alcohol (4.1 mmol) in dichloromethane (5 mL) at 0 °C with stirring. Stirring was continued for 2 h at room temperature, and then the solvent and the excess acetyl chloride were removed by rotary evaporation.

The yield was determined by GC (*9*): 469 mg (65%); MS-EI, *m/z* (intensity in %) 43 (100), 116 (80), 117 (67), 135 (55), 136 (54), 119

(49), 107 (37), 178 (34), 118 (34), 93 (27), 92 (12), 94 (12), 77 (12), 78 (10), 51 (8), 79 (8); MS-CI (methanol), *m/z* (intensity in %) 119 (100; M + $H^+$ − $H_3C$−COOH).

*[2,2,2-$^2H_3$]-Ethyl Butanoate (**d-5**).* In a small tailor-made autoclave (stainless steel, 80 mm, 10 mm i.d., 3 mm wall thickness) with screw cap, PTFE sealing, and glass insert, butanoic acid (20 mmol) and [2,2,2-$^2H_3$]-ethanol (2 mmol) were heated in the presence of sulfuric acid (50 μL) at 80 °C. After 30 min, the mixture was allowed to cool, and after the addition of water (50 mL) followed by diethyl ether (80 mL), the mixture was vigorously shaken. The aqueous phase was removed, and the organic phase was washed with an aqueous sodium carbonate solution (0.5 mol/L; 3 × 100 mL) and water (100 mL). After drying over anhydrous sodium sulfate, the solution was made up to 100 mL. The concentration of the target compound was determined by GC (*9*): yield, 197 mg (83%); MS-EI, *m/z* (intensity in %) 71 (100), 91 (91), 43 (79), 41 (56), 61 (42), 42 (41), 74 (35), 48 (28), 39 (21), 72 (20), 104 (20), 73 (13), 90 (13); MS-CI (methanol), *m/z* (intensity in %) 120 (100; M + $H^+$), 121 (10).

*Methyl [$^2H_5$]-Benzoate (**d-6**).* Following a general procedure for ZnO-catalyzed O-acylations (*11*), [$^2H_5$]-benzoyl chloride (1.8 mmol) was added dropwise to a mixture of methanol (0.5 mL) and zinc oxide (0.62 mmol). After stirring for 30 min at 40 °C, the mixture was extracted with dichloromethane (2 × 25 mL). The combined organic phases were washed with an aqueous sodium hydrogencarbonate solution (10%; 3 × 60 mL) and dried over anhydrous sodium sulfate. Methyl [$^2H_5$]-benzoate was obtained after rotary evaporation.

The yield was determined by GC (*9*): 199 mg (78%); MS-EI, *m/z* (intensity in %) 110 (100), 82 (90), 52 (83), 141 (29), 54 (18), 52 (6), 111 (6), 83 (3), 142 (2); MS-CI (methanol), *m/z* (intensity in %) 142 (100; M + $H^+$).

*Ethyl [$^2H_5$]-Benzoate (**d-7**).* This compound was synthesized as detailed above for the methyl ester using ethanol instead of methanol: yield, 183 mg (66%); MS-EI, *m/z* (intensity in %) 110 (100), 82 (40), 127 (127), 54 (15), 155 (12), 111 (7), 109 (4), 52 (4), 83 (3), 128 (2); MS-CI (methanol), *m/z* (intensity in %), 156 (100; M + $H^+$).

*[$^2H_3$]-Methyl (2R,3S)-2-Hydroxy-3-methylpentanoate (**d-8**).* The compound was synthesized from L-isoleucine (0.45 mmol) and [$^2H_4$]-methanol following the procedure described for the synthesis of the unlabeled compound (**1**).

The yield was determined by GC-FID using butyl lactate as the internal standard: yield, 15 mg (24%); MS-EI, *m/z* (intensity in %) 93 (100), 87 (69), 45 (69), 36 (43), 41 (40), 57 (28), 69 (24), 36 (17), 40 (13), 38 (12), 43 (11), 39 (10), 58 (8); MS-CI (methanol), *m/z* (intensity in %) 150 (100; M + $H^+$).

*[$^2H_3$]-Methyl (2S,3S)-2-hydroxy-3-methylpentanoate (***d-9***)* was synthesized accordingly from D-*allo*-isoleucine: yield, 21 mg (30%); MS-EI, *m/z* (intensity in %) 93 (100), 87 (57), 45 (55), 41 (36), 36 (28), 57 (26), 69 (21), 36 (16), 38 (8), 43 (8), 58 (8), 40 (8), 39 (8); MS-CI (methanol), *m/z* (intensity in %) 150 (100; M + $H^+$).

*[5,5,6,6-$^2H_4$]-Hexanal (**d-11**).* Using Wilkinson catalyst, 5-hexyn-1-ol was deuterated to obtain [5,5,6,6-$^2H_4$]-hexanol, which was subsequently oxidized into [5,5,6,6-$^2H_4$]-hexanal using Dess−Martin−periodinane (*12, 13*).

*(a) [5,5,6,6-$^2H_4$]-Hexanol.* Tris(triphenylphosphin)rhodium(I) chloride (0.27 mmol) in toluene (15 mL) was stirred in a deuterium atmosphere until the deep red suspension turned orange. 5-Hexyn-1-ol (25 mmol) in toluene (15 mL) was added, and the brownish solution was stirred until complete conversion. After dilution with pentane (50 mL), the toluene was removed by column chromatography. To achieve this, the reaction mixture was applied onto silica gel (20 g) filled into a glass column (1.5 cm i.d.), and after flushing with pentane (200 mL), the [5,5,6,6-$^2H_4$]-hexanol was eluted with diethyl ether (100 mL). Residual catalyst was removed by SAFE distillation (*14*), and the solvent was evaporated to yield [5,5,6,6-$^2H_4$]-hexanol in a purity of 99.8%.

*(b) [5,5,6,6-$^2H_4$]-Hexanal.* [5,5,6,6-$^2H_4$]-Hexanol was oxidized into the aldehyde as detailed in (*1*) for the oxidation of (*E*)-3-hexenol into (*E*)-3-hexenal. The resulting solution was made up to 200 mL, and the concentration of the target compound was determined by GC (*9*). As reported earlier (*15*−*17*), the use of tris(triphenylphosphin)rhodium(I) chloride avoided hydrogen−deuterium scrambling during deuteration, frequently observed in heterogeneous catalysis (*18*). Yield, 1.34 g [5,5,6,6-$^2H_4$]-hexanal (54%); MS-EI, *m/z* (intensity in %) 44 (100), 59 (45), 60 (41), 43 (31), 45 (23), 57 (22), 46 (21), 58 (19), 61 (18), 41 (17), 76 (15), 42 (14), 47 (14); MS-CI (methanol), *m/z* (intensity in %) 87 (100; M + $H^+$ − $H_2O$), 105 (93; M + $H^+$), 86 (92; M + $H^+$ − HDO).

*[5,5,6,6,6-$^2H_5$]-(Z)-3-Hexenal (**d-12**).* 2-([5,5,6,6,6-$^2H_5$]-3-Hexyn-1-yloxy)tetrahydro-2*H*-pyran was synthesized from 2-(3-butyn-1-yloxy)-tetrahydro-2*H*-pyran and [$^2H_5$]-ethyl iodide and subsequently hydrolyzed into [5,5,6,6,6-$^2H_5$]-3-hexyn-1-ol (*19*). Using a Lindlar catalyst modified according to ref *20*, [5,5,6,6,6-$^2H_5$]-3-hexyn-1-ol was hydrated to yield [5,5,6,6,6-$^2H_5$]-(*Z*)-3-hexen-1-ol, which was finally converted into [5,5,6,6,6-$^2H_5$]-(*Z*)-3-hexenal using Dess−Martin−periodinane (*13*).

*(a) 2-([5,5,6,6,6-$^2H_5$]-3-Hexyn-1-yloxy)tetrahydro-2H-pyran.* Butyl lithium (15 mmol; 1.5 mL of 10 mmol/mL solution in THF) was added to 2-(3-butyn-1-yloxy)tetrahydro-2*H*-pyran (10 mmol) in anhydrous THF (20 mL) under argon at 0 °C and stirred for 1 h. Then, [$^2H_5$]-ethyl iodide (25 mmol) was added. After 4 h of stirring, an aqueous saturated ammonium chloride solution (10 mL) was added, and the mixture was extracted with diethyl ether (3 × 50 mL). The combined organic phases were washed with an aqueous saturated sodium chloride solution (2 × 10 mL) and dried over anhydrous sodium sulfate to yield 2-([5,5,6,6,6-$^2H_5$]-3-hexyn-1-yloxy)tetrahydro-2*H*-pyran: MS-EI, *m/z* (intensity in %) 85 (100), 86 (17), 41 (9), 83 (9), 57 (8), 67 (8).

*(b) [5,5,6,6,6-$^2H_5$]-3-Hexyn-1-ol.* The solution containing 2-([5,5,6,6,6-$^2H_5$]-3-hexyn-1-yloxytetrahydro-2*H*-pyran was concentrated to 5 mL, and methanol (100 mL) and *p*-toluenesulfonic acid monohydrate (5 mmol) were added. After 2 h of stirring, diethyl ether (100 mL) and water (200 mL) were added. The aqueous phase was removed, and the organic phase was dried over anhydrous sodium sulfate and concentrated by means of a Vigreux column. The [5,5,6,6,6-$^2H_5$]-3-hexyn-1-ol obtained was purified by column chromatography by applying the solution (1 mL) onto a cooled (12 °C) glass column (1 cm i.d.) filled with silica gel (7 g). Elution was performed with pentane (50 mL), followed by pentane/diethyl ether (90:10, 50 mL), pentane/diethyl ether (70:30, 50 mL), pentane/diethyl ether (50:50, 50 mL), and finally diethyl ether (50 mL). The [5,5,6,6,6-$^2H_5$]-3-hexyn-1-ol was eluted between 190 and 230 mL: MS-EI, *m/z* (intensity in %): 73 (100), 72 (35), 71 (31), 55 (16), 103 (14), 56 (13), 44 (12), 45 (10), 57 (10), 42 (9), 41 (8), 74 (8).

*(c) [5,5,6,6,6-$^2H_5$]-(Z)-3-Hexen-1-ol.* After removal of the solvent, [5,5,6,6,6-$^2H_5$]-3-hexyn-1-ol was dissolved in pentane (25 mL) containing quinoline (200 μL) and was hydrated for 2 h in hydrogen atmosphere using a Mn-modified Lindlar catalyst (20 mg) (*20*). The quinoline was removed by column chromatography (for parameters see [5,5,6,6,6-$^2H_5$]-3-hexyn-1-ol above). Pure [5,5,6,6,6-$^2H_5$]-(*Z*)-3-hexen-1-ol was isolated in the effluent between 130 and 140 mL: MS-EI, *m/z* (intensity in %) 105 (100), 86 (94), 43 (91), 74 (75), 57 (59), 71 (54), 69 (47), 45 (46), 70 (46), 68 (44), 60 (43), 42 (36), 87 (28), 41 (26), 85 (24), 58 (24), 72 (21), 40 (21), 59 (20), 46 (20), 47 (19), 56 (18), 39 (16), 55 (13).

*(d) [5,5,6,6,6-$^2H_5$]-(Z)-3-Hexenal.* After removal of the solvent, [5,5,6,6,6-$^2H_5$]-(*Z*)-3-hexen-1-ol was dissolved in dichloromethane (30 mL) and oxidized with 1,1,1-tris(acetyloxy)-1,1-dihydro-1,2-benziodoxol-3-(1*H*)-one as described for (*E*)-3-hexenal (*1*). The concentration of the analyte was determined by GC (*9*) using (*E*)-2-hexenal as the internal standard: yield, 7.5 mg (0.8%); MS-EI, *m/z* (intensity in %) 44 (100), 43 (90), 74 (83), 45 (40), 46 (39), 57 (35), 42 (34), 103 (30), 85 (26), 41 (23), 60 (22), 40 (21), 39 (16), 47 (13), 72 (13), 84 (10), 56 (10), 55 (10), 73 (10), 58 (10); MS-CI (methanol), *m/z* (intensity in %) 85 (100; M + $H^+$ − HDO), 84 (35; M + $H^+$ − $D_2O$), 86 (17; M + $H^+$ − $H_2O$), 104 (17; M + $H^+$).

*[7,7,8,8-$^2H_4$]-trans-4,5-Epoxy-(E)-2-decenal (**d-13**)* was synthesized from [3,3,4,4-$^2H_4$]-hexanal following the procedure described in ref *18*. [3,3,4,4-$^2H_4$]-Hexanal was synthesized from 3-hexyn-1-ol using the same approach as applied for the synthesis of [5,5,6,6-$^2H_4$]-hexanal (**d-11**) from 5-hexyn-1-ol.

**Quantitation of Aroma Compounds by Stable Isotope Dilution Assays.** Whole fruits were blended using a stainless steel blender, and the puree was allowed to stand for exactly 5 min. Then, the labeled standards (0.2−20 μg) dissolved in dichloromethane (50−1000 mL) were added to portions of the puree (0.5−200 g) and further homogenized. With continuous blending and cooling in an ice bath, anhydrous sodium sulfate was added until the mixture became powdery

**Table 1.** Mass Fragments and Calibration Factors Used in the Stable Isotope Dilution Assays for the Quantitation of 17 Aroma Compounds in Guavas

| | | | *m/z*[b] | | |
|---|---|---|---|---|---|
| no. | odorant | isotopic labeling[a] | analyte | internal standard | response factor[c] |
| 1 | 3-sulfanyl-1-hexanol | $^2H_2$ | 117 | 119 | 0.98 |
| 2 | 3-sulfanylhexyl acetate | $^2H_2$ | 117 | 119 | 0.99 |
| 3 | cinnamyl alcohol | $^2H_2$ | 117 | 119 | 0.90 |
| 4 | cinnamyl acetate | $^2H_2$ | 117 | 119 | 0.89 |
| 5 | ethyl butanoate | $^2H_3$ | 117 | 120 | 0.97 |
| 6 | methyl benzoate | $^2H_5$ | 137 | 142 | 0.83 |
| 7 | ethyl benzoate | $^2H_5$ | 151 | 156 | 0.67 |
| 8 | methyl (2*R*,3*S*)-2-hydroxy-3-methylpentanoate | $^2H_3$ | 147 | 150 | 0.98 |
| 9 | methyl (2*S*,3*S*)-2-hydroxy-3-methylpentanoate | $^2H_3$ | 147 | 150 | 0.98 |
| 10 | acetaldehyde[d] | $^{13}C_2$ | 45 | 47 | 0.67 |
| 11 | hexanal | $^2H_4$ | 83 | 87 | 0.54 |
| 12 | (*Z*)-3-hexenal | $^2H_5$ | 81 | 84−86 | 0.98 |
| 13 | *trans*-4,5-epoxy-(*E*)-2-decenal | $^2H_4$ | 139 | 143 | 0.93 |
| 14 | methional (3-(methylthio)propanal) | $^2H_3$ | 105 | 108 | 1.03 |
| 15 | 4-methoxy-2,5-dimethyl-3(2*H*)-furanone | $^2H_3$ | 143 | 146 | 0.91 |
| 16 | 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone | $^{13}C_2$ | 129 | 131 | 1.00 |
| 17 | 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone | $^{13}C_2$ | 129 | 131 | 1.03 |

[a] Labeling in the isotopologues used as internal standards in the stable isotope dilution assays. [b] Mass traces obtained by GC-MS(CI) used for peak area evaluation of analyte and standard, respectively. [c] Response factor determined from reference mixtures of analyte and standard as described in ref *9*. [d] Acetaldehyde was previously not found during AEDA (*1*)*1*, but was detected as an aroma-active compound during headspace analysis of Colombian pink guavas.

and the supernatant organic phase became clear. Then, the mixture was filtered through defatted cotton wool, and the dichloromethane extract was submitted to a SAFE distillation (*14*) at 40 °C.

To measure the influence of time on enzymatic aroma compound formation, samples (1 g) were allowed to stand for 24−600 s (puree) and for 10−600 s (cubes). Then, a saturated aqueous calcium chloride solution (1 mL) was added. To obtain a zero time value, a whole fruit was submersed in calcium chloride solution and cutting was performed below the surface of the solution. Further workup was done as described above.

Depending on the amount of standard added, SAFE distillates were concentrated to a volume of 0.1−10 mL by means of a Vigreux column and a microdistillation apparatus (*21*). Aliquots of the concentrates (0.5−4 μL) were analyzed by means of two-dimensional GC-GC-MS. The system consisted of a Combi PAL autosampler (CTC Analytics, Zwingen, Switzerland), a Trace Ultra GC (Thermo Scientific, Dreieich, Germany), a CP 3800 GC, and a Saturn 2200 mass spectrometer (Varian, Darmstadt, Germany). The Trace GC was equipped with a cold-on-column injector, an FFAP capillary (30 m × 0.32 mm i.d., 0.25 μm film thickness), a moving column stream switching system, and an FID (Thermo Scientific, Dreieich, Germany). The moving column stream switching system was connected to the CP 3800 via an uncoated but deactivated fused silica transfer line (0.32 mm i.d) in a heated (250 °C) hose (Horst, Lorsch, Germany). The GC hosted a cold trap (SGE, Griesheim, Germany) and a DB-1701 column (30 m × 0.25 mm i.d., 0.25 μm film thickness), which was connected to the mass spectrometer. Mass chromatograms were recorded in the MS-CI mode using methanol as the reactant. Analyte concentrations were calculated from the area counts of characteristic mass traces of analyte and standard (**Table 1**) (*9*).

**Quantitation of Acetaldehyde.** Acetaldehyde was determined by stable isotope dilution analysis of static headspace samples. The guava puree (10 g) was diluted with water (50 mL) in a septum-sealed Erlenmeyer flask (200 mL), and [$^{13}C_2$]-acetaldehyde (100 μg) in water (1 mL) was added. After equilibration (30 min), aliquots of the headspace (5 mL) were withdrawn using a gastight syringe and injected onto a DB-5 capillary (25 m × 0.32 mm i.d., 1.2 μm film) held at 0 °C. The column was connected to the mass spectrometer Incos XL (Finnigan MAT, Bremen, Germany) running in the CI mode (methane, 115 eV).

**Odor Thresholds.** Odor thresholds were determined using a panel of 15−20 trained panelists recruited from the Deutsche Forschungsanstalt für Lebensmittelchemie and following the ASTM procedure for the determination of odor and taste thresholds by a forced-choice ascending concentration series method of limits in triangular tests (*22*).

**Aroma Reconstitution.** Appropriate amounts (20−100 μL) of aqueous or ethanolic stock solutions of the odorants were mixed and made up to 1 L with water to yield the same concentrations as determined in the guavas. Final ethanol concentration was kept below 1 g/L, that is, below the odor threshold of ethanol.

**Aroma Profile Tests.** Samples (20 g) were placed into cylindrical ground neck glasses (height = 7 cm; i.d. = 3.5 cm) with lids and were orthonasally evaluated by the sensory panel. Descriptors used were determined in preliminary sensory experiments. Each descriptor used was defined on the basis of the odor of a reference compound dissolved in water at a concentration of 100 times above the respective threshold value. Reference odorants used in the experiments were ethyl butanoate (fruity), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (caramel, sweet), cinnamyl alcohol (flowery), *trans*-4,5-epoxy-(*E*)-2-decenal (metallic), 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (seasoning-like), (*Z*)-3-hexenal (grassy), acetaldehyde (fresh), and 3-sulfanyl-1-hexanol (grapefruit). Assessors were asked to rate each descriptor in the samples presented on a seven -point scale from 0 to 3, with 0 = not detectable, 1 = weak, 2 = moderate, and 3 = strong.

**Omission Tests.** Models were prepared as detailed above. In triangular tests with forced choice, models in which one odorant was omitted were evaluated against two samples of the complete model.

## RESULTS AND DISCUSSION

**Concentrations of the Most Aroma-Active Compounds in Guavas.** Sixteen odorous compounds that had shown high FD factors in the previous study (*1*), namely, 4-methoxy-2,5-dimethyl-3(2*H*)-furanone, 3-sulfanylhexyl acetate, 3-sulfanyl-1-hexanol, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone, (*Z*)-3-hexenal, *trans*-4,5-epoxy-(*E*)-2-decenal, cinnamyl alcohol, ethyl butanoate, hexanal, methional, cinnamyl acetate, methyl benzoate, methyl (*R*,*S*)-2-hydroxy-3-methylpentanoate, ethyl benzoate, and methyl (*S*,*S*)-2-hydroxy-3-methylpentanoate, were quantified by stable isotope dilution assays. In addition, acetaldehyde, which was found in this study as an additional aroma-active compound during static headspace GC sniffing analyses applied on a fresh pink Colombian guava puree (data not shown), was included in the investigations.

For each of the 17 odorants selected (**Table 1**) a stable isotopologue was synthesized (**Figure 1**) and used as internal standard in the quantitations.

The results of the quantitation experiments (**Table 2**) revealed concentrations ranging from the lower micrograms per kilogram area to several milligrams per kilogram. In particular, high amounts were found for (*Z*)-3-hexenal (6.9 mg/kg), acetaldehyde (2.4 mg/kg), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (1.4 mg/kg), and cinnamyl alcohol (1.3 mg/kg), whereas methional (1.3 μg/kg) was 1000 times lower. To estimate the aroma potency of the individual guava odorants, their concentrations were correlated with the respective odor thresholds using the odor activity value (OAV) concept (*23*).

The green grassy smelling (*Z*)-3-hexenal showed the highest OAV (**Table 3**), exceeding its threshold by a factor of 57000. Second in rank was the grapefruit-like smelling 3-sulfanyl-1-hexanol with an OAV of 9300. High OAVs were also calculated for 3-sulfanylhexyl acetate (black currant-like; OAV 570), hexanal (green, grassy; OAV 360), and ethyl butanoate (fruity; OAV 170). Somewhat lower OAVs were determined for acetaldehyde (fresh, pungent), *trans*-4,5-epoxy-(*E*)-2-decenal (metallic), 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (caramel, sweet), cinnamyl alcohol (floral), and methyl (2*S*,3*S*)-2-hydroxy-3-methylpentanoate (fruity). On the other hand, the concentrations of cinnamyl acetate, methional,

**Table 2.** Concentrations of Aroma-Active Compounds in Colombian Pink Guavas

| odorant | concn (μg/kg) | no. of replicates | SD (%) |
|---|---|---|---|
| (*Z*)-3-hexenal | 6890 | 5 | 4 |
| acetaldehyde | 2440 | 3 | 45 |
| 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone | 1420 | 5 | 4 |
| cinnamyl alcohol | 1290 | 5 | 11 |
| cinnamyl acetate | 862 | 5 | 9 |
| hexanal | 857 | 3 | 6 |
| 3-sulfanyl-1-hexanol | 555 | 2 | 2 |
| ethyl butanoate | 126 | 3 | 13 |
| methyl (2*S*,3*S*)-2-hydroxy-3-methylpentanoate | 27.2 | 3 | 12 |
| *trans*-4,5-epoxy-(*E*)-2-decenal | 16.8 | 3 | 24 |
| 3-sulfanylhexyl acetate | 11.4 | 2 | 5 |
| methyl (2*R*,3*S*)-2-hydroxy-3-methylpentanoate | 9.7 | 3 | 16 |
| 4-methoxy-2,5-dimethyl-3(2*H*)-furanone | 9.4 | 7 | 8 |
| ethyl benzoate | 7.2 | 2 | 0 |
| 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone | 4.3 | 5 | 39 |
| methyl benzoate | 2.6 | 3 | 14 |
| methional | 1.3 | 3 | 12 |

**Table 3.** Orthonasal Odor Thresholds and Odor Activity Values (OAV) of Aroma-Active Compounds in Guavas

| odorant | odor quality | threshold (μg/kg of water) | OAV[a] |
|---|---|---|---|
| (*Z*)-3-hexenal | grassy | 0.12 | 57000 |
| 3-sulfanyl-1-hexanol[b] | grapefruit | 0.06 | 9300 |
| 3-sulfanylhexyl acetate[b] | black currant | 0.02 | 570 |
| hexanal | grassy | 2.4 | 360 |
| ethyl butanoate | fruity | 0.76 | 170 |
| acetaldehyde | fresh, pungent | 25 | 98 |
| *trans*-4,5-epoxy-(*E*)-2-decenal | metallic | 0.22 | 76 |
| 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone | caramel, sweet | 40 | 36 |
| cinnamyl alcohol | floral | 77 | 17 |
| methyl (2*S*,3*S*)-2-hydroxy-3-methylpentanoate | fruity | 2.4 | 11 |
| cinnamyl acetate | floral | 150 | 5.7 |
| methional | cooked potato | 0.43 | 3.1 |
| 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone | seasoning-like | 1.7 | 2.5 |
| methyl (2*R*,3*S*)-2-hydroxy-3-methylpentanoate | fruity | 13 | 0.7 |
| ethyl benzoate | violet, floral | 53 | $\ll 1$ |
| 4-methoxy-2,5-dimethyl-3(2*H*)-furanone | caramel, sweet | 160 | $\ll 1$ |
| methyl benzoate | violet, floral | 73 | $\ll 1$ |

[a] OAV = odor activity value = concentration divided by threshold. [b] Because enantiomeric distribution in guavas was close to 1:1, a racemic mixture was used for threshold determination.

and 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone were only slightly above their threshold values. Because the OAVs of methyl (2*R*,3*S*)-2-hydroxy-3-methylpentanoate, ethyl benzoate, 4-methoxy-2,5-dimethyl-3(2*H*)-furanone, and methyl benzoate were below 1, these compounds are assumed not to contribute to the overall aroma.

**Aroma Reconstitution Experiments.** It is a well-known phenomenon that the overall aroma of a mixture of odor-active compounds cannot be predicted. The reason for this is the fact that the signals caused by the single interactions of the odorants with the respective olfactory receptor neurons are combined by the brain to generate the overall aroma perception. Thus, the re-engineering of the aroma by using the natural concentrations of the single odorants in a so-called "aroma reconstitute" is the only available method to confirm that the identifications and quantitations have led to the original blueprint of an aroma. Thus, an aqueous solution containing the 13 odorants found to exceed their respective thresholds (cf. **Table 3**) in the concentrations determined (cf. **Table 2**) was compared to a fruit puree

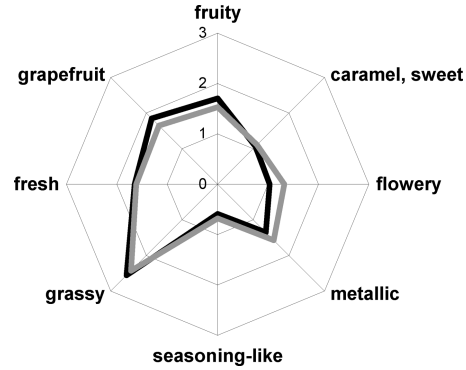


**Figure 2.** Aroma profile of the Colombian pink guava puree model (gray) in comparison with the original fruit puree (black).

prepared from fresh guavas by a sensory panel in an aroma profile test. Despite the simplified matrix not including any nonvolatile guava constituents, such as carbohydrates, acids, or pectin, the results (**Figure 2**) showed a good agreement between aroma model solution and the original guava puree. Both were characterized by a strong green, grassy note, a moderate grapefruit-like, some fruity and fresh notes, and rather weak sweet, flowery, and metallic notes.

**Changes during Fruit Tissue Disruption.** Despite the good agreement of the aroma profiles of the model and the guava puree, the panelists suggested that the green grassy note was too predominant when compared to the perception detected during guava fruit consumption. According to the OAVs, (*Z*)-3-hexenal should undoubtedly be responsible for the green, grassy odor note. The odorant is known to be formed upon plant tissue disruption from α-linolenic acid (*24*) via the enzymatically formed 13-(*S*)-hydroperoxide, which is finally cleaved by a hydroperoxide lyase to yield (*Z*)-3-hexenal. Therefore, it might be suspected that the homogenization of the guavas before workup was responsible for the high (*Z*)-3-hexenal concentration and, thus, for the high intensity of the green odor note in the resulting puree. On the contrary, during consumption of whole fruits (*Z*)-3-hexenal formation might be lower, either due to the smaller time window of modification or a smaller degree of tissue disruption during chewing. To follow these considerations, the time course of (*Z*)-3-hexenal formation in a freshly prepared guava puree in comparison to fruit cubes of approximately 0.5 cm edge length derived from the same fruit was determined. To stop enzymatic reactions, the samples were poured into an aqueous saturated calcium chloride solution, which is known to inhibit the enzymes of the lipoxygenase pathway (*25*).

The results showed clear differences between (*Z*)-3-hexenal formation in the puree and in the cubes, respectively (**Figure 3**). As expected, the zero value, representing the (*Z*)-3-hexenal content in the intact fruit tissue, was quite low. However, in the puree, the (*Z*)-3-hexenal concentration quickly increased to 21 mg/kg after 24 s, just the time span needed for blending the guavas. Upon standing, (*Z*)-3-hexenal concentration in the puree showed a further increase, reaching 42 mg/kg after 10 min. Compared to the first quantitations (**Table 2**), showing concentrations in the puree of 6.9 mg/kg, in this experiment (*Z*)-3-hexenal formation was considerably higher, yielding almost 28 mg after 5 min.

A possible explanation for this observation could be a different ripening history of the two fruit batches. Fruits used to obtain the values listed in **Table 2** were picked in Colombia in a full ripe state and transported to Germany by air, whereas the fruit used for the time course was purchased in Germany

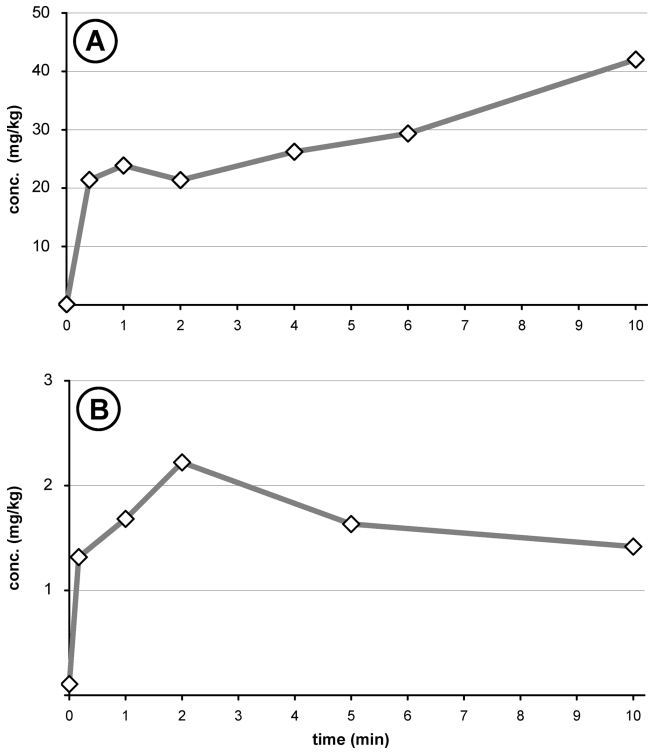


**Figure 3.** Time course of (*Z*)-3-hexenal formation in guava puree (**A**) and guava cubes (**B**) derived from the same fruit.

**Table 4.** Concentrations and Odor Activity Values (OAV) of Aroma-Active $C_6$-Aldehydes in Guava Cubes

| odorant | concn ($\mu$g/kg) | threshold ($\mu$g/kg of water) | OAV[a] |
|---|---|---|---|
| (*Z*)-3-hexenal | 1630 | 0.12 | 14000 |
| hexanal | 652 | 2.4 | 270 |
| (*E*)-2-hexenal | 67 | 110 | 0.6 |

[a] OAV = odor activity value (concentration divided by odor threshold).

from a commercial source and therefore might have been harvested at an earlier stage of maturity.

In comparison to the puree, (*Z*)-3-hexenal formation in the cubes was by far lower. As in the puree, the initial increase was fast, resulting in 1.3 mg/kg (*Z*)-3-hexenal after 10 s. A further increase was then observed until a maximum of 2.2 mg/kg was reached after 2 min. Then, the (*Z*)-3-hexenal concentration slowly decreased to 1.4 mg/kg after 10 min. These experiments clearly showed that (*Z*)-3-hexenal formation taking place upon crushing of guava fruits is a very rapid process and that the final (*Z*)-3-hexenal concentrations are highly dependent on the degree of tissue disruption.

**Aroma Reconstitution Based on Data for Fruit Cubes.** The aroma reconstitution experiment was repeated, but using the concentrations for (*Z*)-3-hexenal and hexanal obtained from the fruit cubes after 5 min (**Table 4**) instead of the comparably higher values found in the puree (**Table 2**). Despite the clearly lower concentration, (*Z*)-3-hexenal was still the compound with the highest odor activity value (14000) and by far more potent than hexanal, exhibiting the same green, grassy odor characteristics, but at a substantially lower OAV of 270. (*E*)-2-Hexenal, which is formed from (*Z*)-3-hexenal by an enzymatic isomerization reaction (*24*), was not present in aroma-active amounts (OAV < 1) and was therefore omitted.

An aqueous solution containing the 13 odorants (*Z*)-3-hexenal, 3-sulfanyl-1-hexanol, 3-sulfanylhexyl acetate, hexanal, ethyl butanoate, acetaldehyde, *trans*-4,5-epoxy-(*E*)-2-decenal, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, cinnamyl alcohol, methyl (2*S*,3*S*)-2-hydroxy-3-methylpentanoate, cinnamyl acetate, methional, and 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone was compared in an aroma profile test to an original fruit sample, which consisted of guava cubes. The concentrations of (*Z*)-3-hexenal and hexanal were reduced as indicated in **Table 4**, but all other compounds were used as given in **Table 2**. The results (**Figure 4**) again revealed a good match between the aroma model solution and the fruit sample. As expected, the grassy note was now assessed much lower as compared to the aroma profile of the puree. In contrast, the grapefruit, fruity, and sweet notes were rated higher. Obviously these notes were suppressed to a certain degree in the puree and the reconstitute thereof by the overwhelming green, grassy odor of (*Z*)-3-hexenal. Generally, the orthonasal profiles of the guava fruit cubes and its reconstituted model were more balanced than those of the puree samples and were close to the aroma perception during consumption of guava fruits. However, our approach to the use of fruit cubes as sample for the quantitation of the aroma-active $C_6$-aldehydes was somewhat empirical. Therefore, further investigations into the release behavior of these compounds during fruit consumption, for example, by online measurements during chewing, will be performed.

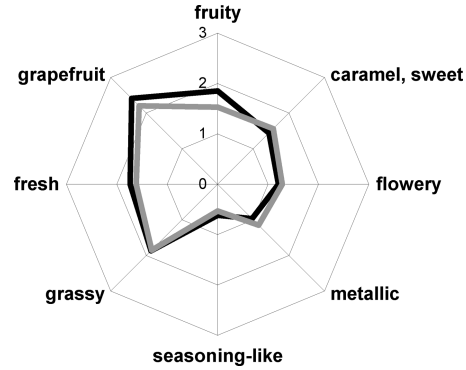


**Figure 4.** Aroma profile of the Colombian pink guava cubes model (gray) in comparison with the original fruit cubes (black).

**Omission Tests.** Omission tests can be applied to assess the contribution of individual odorants to the overall aroma (*26*) and, thus, to define the key aroma compounds. In the present case, omission tests were performed using the aroma reconstitute of the guava cubes. In 13 independent experiments all 13 odorants were singly omitted from the model, and these incomplete models were evaluated against the complete model in orthonasal triangular tests by a sensory panel.

The results (**Table 5**) revealed significant differences in the overall aroma when either (*Z*)-3-hexenal, 3-sulfanyl-1-hexanol, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 3-sulfanylhexyl acetate, hexanal, ethyl butanoate, cinnamyl acetate, or methional, respectively, was missing in the model. The most noticeable aroma differences (level of significance = 0.1%) were perceived when (*Z*)-3-hexenal or 3-sulfanyl-1-hexanol was omitted, which was not surprising because these compounds also showed by far the highest OAVs. Omission of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, 3-sulfanylhexyl acetate, and hexanal was detected at a level of significance of 1%, also corresponding to their high OAVs. However, the clear perception of a difference after omission of hexanal in the presence of an outstanding amount of (*Z*)-3-hexenal is somewhat unclear. Yet at a level of significance of 5%, the omission of ethyl butanoate, cinnamyl acetate, and methional was detected, indicating also an essential aroma contribution of these odorous compounds. In contrast, omitting cinnamyl alcohol, *trans*-4,5-epoxy-(*E*)-2-decenal, ac-

**Table 5.** Results of Omission Tests Applied on the Aroma Reconstitute of Guava Cubes

| odorant omitted | no. of panelists | correct answers | level of significance (%) |
|---|---|---|---|
| (*Z*)-3-hexenal | 19 | 16 = 84% | 0.1 |
| 3-sulfanyl-1-hexanol | 16 | 12 = 75% | 0.1 |
| 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone | 15 | 11 = 73% | 1 |
| 3-sulfanylhexyl acetate | 14 | 10 = 71% | 1 |
| hexanal | 16 | 11 = 69% | 1 |
| ethyl butanoate | 14 | 9 = 64% | 5 |
| cinnamyl acetate | 16 | 10 = 63% | 5 |
| methional | 16 | 9 = 56% | 5 |
| cinnamyl alcohol | 14 | 7 = 50% | ns[a] |
| *trans*-4,5-epoxy-(*E*)-2-decenal | 19 | 8 = 42% | ns |
| acetaldehyde | 16 | 4 = 25% | ns |
| methyl (2*S*,3*S*)-2-hydroxy-3-methylpentanoate | 16 | 4 = 25% | ns |
| 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone | 19 | 3 = 16% | ns |

[a] Not significant.

etaldehyde, methyl (2*S*,3*S*)-2-hydroxy-3-methylpentanoate, or 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone did not result in a significant aroma change, although, for example, acetaldehyde and *trans*-4,5-epoxy-(*E*)-2-decenal showed rather high OAVs. Obviously, in the complex mixture, their aroma is somewhat suppressed.

In conclusion, the omission tests clearly elucidated the key aroma compounds of guava fruits. (*Z*)-3-Hexenal and, to a lower extent, hexanal are responsible for the green, grassy odor note, whereas 3-sulfanyl-1-hexanol and 3-sulfanylhexyl acetate account for the sulfury, tropical note and 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, ethyl butanoate, and cinnamyl acetate predominantly make up the sweet, fruity, and flowery odor characteristics.

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