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Identification of the Key Aroma Compounds in Cocoa Powder Based on Molecular Sensory Correlations

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Isolation of the volatile fraction from cocoa powder (50 g; 20% fat content) by a careful extraction/distillation process followed by application of an aroma extract dilution analysis revealed 35 odor-active constituents in the flavor dilution (FD) factor range of 8−4096. Among them, 4-hydroxy-2,5-dimethyl-3(*2H*)-furanone (caramel-like), 2- and 3-methylbutanoic acid (sweaty, rancid), dimethyl trisulfide (cooked cabbage), 2-ethyl-3,5-dimethylpyrazine (potato-chip-like), and phenylacetaldehyde (honey-like) showed the highest FD factors. Quantitation of 31 key odorants by means of stable isotope dilution assays, followed by a calculation of their odor activity values (OAVs) (ratio of concentration to odor threshold) revealed OAVs > 100 for the five odorants acetic acid (sour), 3-methylbutanal (malty), 3-methylbutanoic acid, phenylacetaldehyde, and 2-methylbutanal (malty). In addition, another 19 aroma compounds showed OAVs > 1. To establish their contribution to the overall aroma of the cocoa powder, these 24 compounds were added to a reconstructed cocoa matrix in exactly the same concentrations as they occurred in the cocoa powder. The matrix was prepared from deodorized cocoa powder, which was adjusted to 20% fat content using deodorized cocoa butter. The overall sensory evaluation of this aroma recombinate versus the cocoa powder clearly indicated that the 24 compounds represented the typical sweet, cocoa-like odor of the real sample.

KEYWORDS: Cocoa powder; aroma extract dilution analysis; stable isotope dilution assay; aroma recombination; sensomics

INTRODUCTION

The seeds of the cocoa tree (cocoa beans; *Theobroma cacao* L.) have been used since ancient times in the preparation of tasty foods, and today >3 million metric tons of cocoa beans are harvested (*1*). The characteristic aroma of cocoa is developed as a result of a crude fermentation procedure applied to the fresh seeds, followed by drying and roasting, and different temperature/time regimens are known to significantly influence the type of overall aroma formed (*2*).

For chocolate production, roasted cocoa nibs (broken beans) are ground and liquified by heating to obtain the cocoa liquor, which is then separated into cocoa powder and cocoa butter by pressing. If the production of cocoa powder is intended, the press cake obtained after the above procedure can be treated with alkali or treatment with sodium or potassium carbonate is done directly on either the fermented beans or the cocoa mass before pressing. The alkaline treatment was invented by van Houten, and this process, known as "Dutching", is mainly intended to partially remove acetic acid formed during fermentation and, also, to develop a darker color and to improve the suspension of the cocoa particles in aqueous media. Cocoa powders are mainly used in the preparation of milk-based beverages and for confectionery purposes (*2*).

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The attractive aroma of roasted cocoa has stimulated research on this topic since 1912, when Bainbridge and Davies (*3*) identified linalool and several esters as the main volatile constituents in an extract prepared from ≈2 tons of roasted cocoa beans. In the following decades, comprehensive studies performed by research groups at Firmenich (*4*−*6*) and by van Praag et al. (*7*), van der Wal et al. (*8*), and Vitzthum et al. (*9*) have led to the identification of hundreds of volatile compounds in different types of cocoa, and, today, >550 volatile constituents are known (*10*). In particular, aldehydes formed by Strecker degradation of amino acids, such as methylpropanal, 2- and 3-methylbutanal, and phenylacetaldehyde as well as pyrazines were quite early suggested as important contributors to the cocoa aroma (*7*); however, this has not yet been proven by a systematic combination of sensory experiments and molecular sensory science.

By application of gas chromatography−olfactometry (GC-O) in combination with an aroma extract dilution analysis (AEDA), Schnermann and Schieberle (*11*) were the first to characterize the most odor-active compounds in a commercial cocoa mass. The highest flavor dilution (FD) factors (for definition see review in ref *12*) were determined for 3-methylbutanal, ethyl 2-methylbutanoate, hexanal, 2-isopropyl-3-methoxypyrazine, 2-ethyl-3,5-dimethylpyrazine, and 2- and 3-methylbutanoic acid. Furthermore, 2-methyl-3-(methyldithio)-

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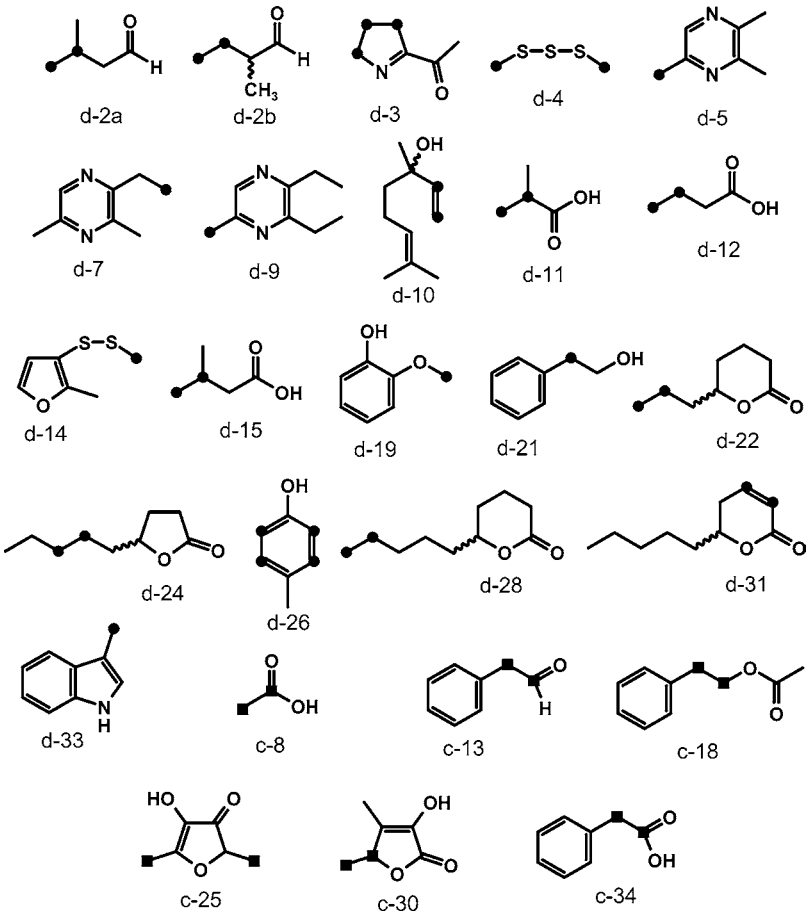

Figure 1. Structures of isotopically labeled internal standards used in the stable isotope dilution assays: (●) 100% deuterium label at the respective carbon atom; (■) 100% carbon-13 label.

furan, eliciting a meat-like odor, was identified for the first time as a previously unknown cocoa constituent.

Methods using GC-O in combination with dilution to odor threshold methods, such as AEDA (*12*) or CHARM analysis (*13*) as well as other GC-O-related techniques such as olfactometry global analysis (*14*), OSME (*15*), or the finger span matching method (*16*) are all useful tools to locate odor-active constituents in complex mixtures of odorless volatile constituents. However, all methods use separation of volatiles by gas chromatography prior to the sniffing procedure and, thus, always the total amount of each volatile present in the extract is volatilized for human perception. However, in a food, the amount of a volatile available for aroma perception depends on its volatility from the respective food matrix. Therefore, results obtained by such GC-O-based techniques must be confirmed by precise quantitative measurements (*12*). Furthermore, only by an overall sensory evaluation of a mixture of the key odorants in their "natural" concentrations, a method called aroma recombination (*12*), can the challenges resulting from masking or enhancing effects at the human odorant receptors be addressed. This concept, which can be assigned as "molecular sensory correlation" or "sensomics", respectively, provides the final confirmation that the correct molecules causing the aroma perception at the human olfactory bulb have been identified. Because this approach has not been applied on either roasted cocoa or cocoa powder, the purpose of this study was to characterize the key aroma compounds in a commercial cocoa powder by (i) application of an AEDA, (ii) quantitations based on isotope dilution assays, and, finally, (iii) an aroma recombination. The investigation is part of an ongoing study on the influence of fermentation and processing on cocoa aroma.

MATERIALS AND METHODS

Materials. Partially defatted cocoa powder (20% fat content) was obtained from a cocoa powder manufacturer and was stored at 4 °C before analysis. According to the manufacturer, the cocoa mass had been treated with alkali to obtain the "dutched" cocoa powder. Fresh samples of the same batch were obtained every 6 months.

Reference Odorants. The following reference compounds were obtained from the sources given in parentheses: benzyl acetate, (*R*/*S*)-δ-decalactone, (*R*/*S*)-γ-decalactone, 2,3-diethyl-5-methylpyrazine, dimethyl trisulfide, 2-ethyl-3,5-dimethylpyrazine, 3-ethylphenol, 3-hydroxy-4,5-dimethyl-2-(5*H*)-furanone, 2-methoxy-4-propenylphenol, 4-hydroxy-2,5-dimethyl-3-(2*H*)-furanone, 4-methylphenol, (*R*/*S*)-linalool, 2-methoxy-3-isobutylpyrazine, (*R*/*S*)-2-methylbutanal, (*R*/*S*)-2-methylbutanoic acid, 3-methylbutanoic acid, 3-methylindole, methylpropanal, methylpropanoic acid, (*R*/*S*)-γ-nonalactone, (*R*/*S*)-δ-octalactone, 1-octen-3-one, phenylacetaldehyde, phenylacetic acid, 2-phenylethanol, 2-phenylethyl acetate, benzyl alcohol, 2-methyl-3-furanthiol, and 2,3,5-trimethylpyrazine (Sigma-Aldrich, Taufkirchen, Germany); butanoic acid, acetic acid, and vanillin (Merck, Darmstadt, Germany); 2-methoxyphenol (Serva, Heidelberg, Germany); 3-methylbutanal (Lancaster, Mühlheim, Germany). The mention of a supplier does not mean that this is the only commercial source of the respective chemical. The name of the company is indicated for scientific purposes only.

The following reference odorants were synthesized according to the literature cited: 2-acetyl-1-pyrroline (*17*) and δ-decenolactone (*18*). 2-Methyl-3(methyldithio)furan was synthesized following the procedure described below for the isotopically labeled compound, but using methyl iodide instead of [$^2H_3$]-methyl iodide.

Isotopically Labeled Internal Standards. The isotopically labeled internal standards used, labeled either with deuterium or with carbon-13, were synthesized as described in the references given in parentheses (numbering refers to **Figure 1**) and are also correlated to the numbers of the respective odorants described in the tables: d-**2a** (*19*; M.

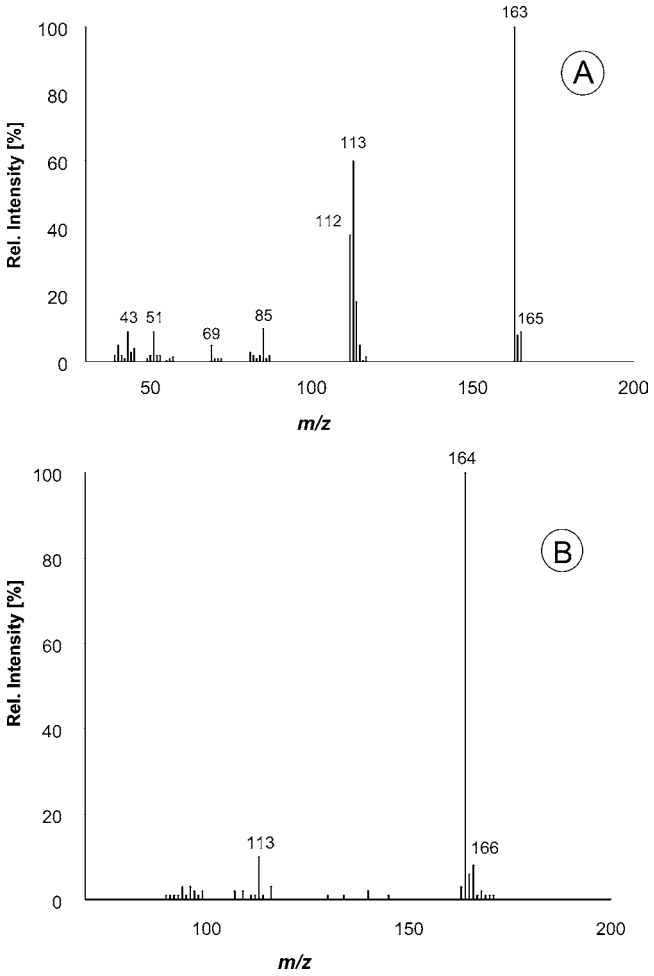

Figure 2. Mass spectra of [$^2H_3$]-2-methyl-3-(methyldithio)furan: (**A**) MS-EI; (**B**) MS-CI.

Christlbauer, unpublished results); d-**2b** (*19, 20*); d-**3** (*21*); d-**4** (*22*); d-**5** (M. Czerny, unpublished results); d-**7** and d-**9** (*23*); d-**12** (*24*); d-**14** (see below); d-**15** (*25*); d-**19** (*23*); d-**21** (*26*); d-**22** (*24*); d-**24** (*27*); d-**28** (*24*); d-**31** (see below); d-**33** (*28*); c-**13** (*26*); c-**18** (M. Czerny, unpublished results); c-**25** (*29*) and c-**30** (*30*). [$^2H_2$]-Methylpropanoic (d-**11**; **Figure 1**) acid was synthesized using deuterium instead of hydrogen in the procedure described recently (*31*). [$^2H_7$]-4-Methylphenol (d-**26**), [$^2H_3$]-acetic acid (d-**8**), and [$^{13}C_2$]-phenylacetic acid (c-**34**) were purchased from Sigma Aldrich (Taufkirchen, Germany).

*[$^2H_3$]-2-Methyl-3-(methyldithio)furan (d-**14**).* In the first step a Bunte salt was formed by treating sodium thiosulfate (0.3 mmol), dissolved in 10 mL of water, with [$^2H_3$]-methyliodide (0.3 mmol) dissolved in 10 mL of ethanol for 6 h at 50 °C. After this had cooled to room temperature, a solution of 2-methyl-3-furanthiol (0.3 mmol) in 25 mL of diethyl ether was added and stirred for 2 h. The organic layer was separated, and the aqueous layer was washed with diethyl ether (3 × 25 mL). The combined organic phases were washed with aqueous sodium carbonate (0.5 mol/L, 3 × 25 mL) and subsequently with saturated aqueous NaCl (25 mL) and then dried over anhydrous sodium sulfate. The solution was concentrated to 1 mL and applied onto a water-cooled column (25 cm × 1.5 cm) filled with a slurry of silica gel (30 g) in pentane. The column was first flushed with pentane (150 mL), followed by a mixture of pentane/diethyl ether (150 mL, 95:5, v/v). The fraction eluting between 100 and 150 mL was concentrated to 1 mL and separated by column chromatography (100 × 30 mm) on a reversed phase stationary HPLC phase (LiChroprep RP18; 20−40 μm, Merck) using mixtures of aqueous ammonium formate (10 mmol/L, pH 8.2) and methanol (50:50, 40:60, 30:70, 20:80, 15:85 v/v, 100 mL each) as the mobile phases. [$^2H_3$]-2-Methyl-3-(methyldithio)furan was eluted in the final fraction. The solutions containing the target compound were combined, and aqueous saturated NaCl (25 mL) and diethyl ether were added with vigorous stirring (50 mL). The aqueous organic phase was separated and extracted four times with diethyl ether (50 mL each). The combined organic phases were dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure. The residue was dissolved in ethanol.

The structure of the target compound was confirmed by mass spectral analysis (**Figure 2A**,**B**) and ^{1}H NMR measurements. In comparison to the ^{1}H NMR of the unlabeled compound [^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.39 (s, 3H), 2.45 (s, 3H), 6.43 (dd, 1H, J = 1.96), 7.28 (dd, 1H, J = 1.96)], as expected, the singlet at δ 2.39 (−S−S−CH_3) was missing.

The concentration of the target compound was determined using methylfuran as the internal standard, a compound with a comparable number of effective carbon atoms (*32*).

*[$^2H_2$]-δ-Decenolactone (d-**31**).* The labeled lactone was synthesized following the route shown in **Figure 3**. To a stirred solution of 1-heptene (0.04 mol) in dichloromethane (80 mL) was added dropwise a solution of 3-chloroperbenzoic acid (0.04 mol in 60 mL of dichloromethane). After 3 h, the solution was treated with aqueous sodium hydroxide (10%; 100 mL) followed by tap water (100 mL). The organic phase was dried over anhydrous sodium sulfate, and the 1,2-epoxyheptane formed was isolated by distillation (bp 120 °C; yield 95%).

A solution of *tert*-butyl propionate (0.03 mol/L) in tetrahydrofuran (60 mL) was cooled to −78 °C and *n*-butyl-lithium (0.03 mol) was added. After 15 min of stirring, 1,2-epoxyheptane dissolved in tetrahydrofuran (30 mL) was added followed by ethereal boron trifluoride (3.9 mL). After 90 min of stirring at −78 °C, aqueous potassium dihydrogenphosphate (10%; 20 mL) was added while the solution was allowed to reach room temperature. The organic phase was removed, and the aqueous phase was extracted with diethyl ether/pentane 1:1 (total volume 90 mL). The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated to ≈1 mL by distilling off the solvent using a Vigreux column (60 cm × 1 cm). The target compound was isolated by flash chromatography using silica gel [30 g in a water-cooled glass column (30 cm × 1.5 cm)] and ethyl acetate/pentane (20:80; 250 mL) as the eluent. The volume was reduced to ≈10 mL using a Vigreux column and made up to 50 mL with ethyl acetate. After the addition of Lindlar catalyst (500 mg) and quinoline (10 mg), the solution was stirred under an atmosphere of deuterium

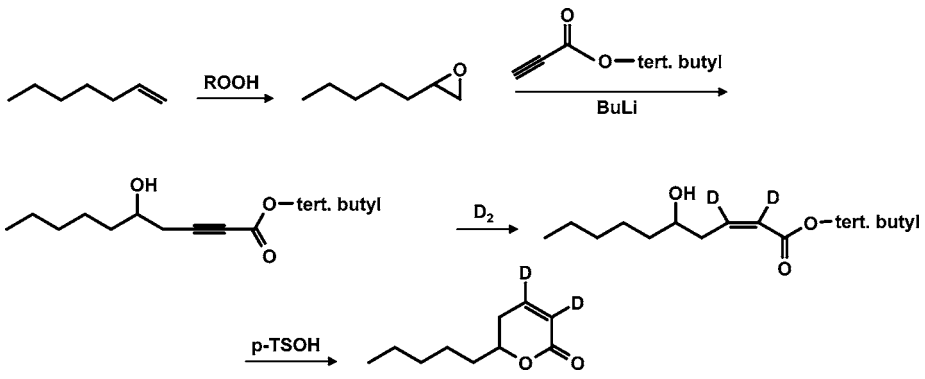

Figure 3. Synthetic route used in the preparation of [$^2H_2$]-δ-decenolactone.

Table 1. Selected Ions and Response Factors Used in the Stable Isotope Dilution Assays

odorant[a]	ion (*m*/*z*)	internal standard	no.	ion (*m*/*z*)	RF[b]
2-methylbutanal[c]	69	[$^2H_2$]-2-methylbutanal	d-**2a**	70 + 71	0.78
3-methylbutanal[c]	69	[$^2H_2$]-3-methylbutanal	d-**2b**	71	0.69
2-acetyl-1-pyrroline[c]	112	[$^2H_{2-5}$]-2-acetyl-1-pyrroline	d-**3**	114–117	0.83
dimethyl trisulfide	127	[$^2H_6$]-dimethyl trisulfide	d-**4**	133	1.0
2,3,5-trimethylpyrazine	123	[$^2H_3$]-2,3,5-trimethylpyrazine	d-**5**	126	0.93
2-ethyl-3,6-dimethylpyrazine	137	[$^2H_3$]-2-ethyl-3,5-dimethylpyrazine	d-**7**	140	0.93
2-ethyl-3,5-dimethylpyrazine	137	[$^2H_3$]-2-ethyl-3,5-dimethylpyrazine	d-**7**	140	0.95
acetic acid[d]	61	[$^2H_3$]-acetic acid	d-**8**	64	0.87
2,3-diethyl-5-methylpyrazine[c]	151	[$^2H_3$]-2,3-diethyl-5-methylpyrazine	d-**9**	154	0.95
linalool[c]	137	[$^2H_2$]-linalool	d-**10**	139	1.0
methylpropanoic acid[d]	89	[$^2H_2$]-methylpropanoic acid	d-**11**	91	1.0
butanoic acid[d]	103	[$^2H_2$]-butanoic acid	d-**12**	105	0.85
2-methyl-3-(methyldithio)furan[c]	161	[$^2H_3$]-2-methyl-3-(methyldithio)furan	d-**14**	164	0.90
3-methylbutanoic acid[d]	103	[$^2H_2$]-3-methylbutanoic acid	d-**15**	105	0.67
2-methoxyphenol[d]	125	[$^2H_3$]-2-methoxyphenol	d-**19**	128	0.98
2-phenylethanol[d]	105	[$^2H_2$]-2-phenylethanol	d-**21**	107	0.94
δ-octalactone	143	[$^2H_2$]-δ-octalactone	d-**22**	145	0.88
δ-octenolactone	141	[$^2H_2$]-δ-octalactone	d-**22**	145	0.98
γ-nonalactone	157	[$^2H_2$]-γ-nonalactone	d-**24**	159	0.93
4-methylphenol[c]	109	[$^2H_7$]-4-methylphenol	d-**26**	115 + 116	1.0
γ-decalactone	171	[$^2H_2$]-δ-decalactone	d-**28**	173	0.89
δ-decalactone	171	[$^2H_2$]-δ-decalactone	d-**28**	173	0.93
δ-decenolactone	169	[$^2H_2$]-δ-decenolactone	d-**31**	171	0.91
3-methylindole[c]	132	[$^2H_3$]-3-methylindole	d-**33**	135	0.76
phenylacetaldehyde	121	[$^{13}C_2$]-phenylacetaldehyde	c-**13**	123	1.0
2-phenylethyl acetate[d]	166	[$^{13}C_2$]-2-phenylethyl acetate	c-**18**	168	1.0
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone[c,d]	129	[$^{13}C_2$]-4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	c-**25**	131	1.0
3-hydroxy-4,5-dimethyl-2(5*H*)-furanone[c,d]	129	[$^{13}C_2$]-3-hydroxy-4,5-dimethyl-2(5*H*)-furanone	c-**30**	131	1.0
2-phenylacetic acid[d]	137	[$^{13}C_2$]-2-phenylacetic acid	c-**34**	139	1.0

[a] Compounds were quantified after separation on an OV 1701 GC stationary phase by mass spectrometry in MS-CI mode. [b] The response factor (RF) was determined as reported previously (*29*). [c] The quantitation was performed by means of TD-GC-MS. [d] The quantitation was performed on the FFAP column.

for 2 h. The catalyst was filtered off, and the solvent was partially removed to obtain a final volume of ≈5 mL. After the addition of water-saturated toluene (20 mL) and toluenesulfonic acid (0.2 g), the solution was refluxed for 90 min. After cooling to room temperature, the solution was treated with aqueous sodium bicarbonate (0.5 mol/L; 30 mL), washed with tap water (30 mL), and dried over anhydrous Na_2SO_4. An aliquot of the pale yellow solution (2 mL) was placed on the top of a water-cooled glass column filled with a slurry of purified silica gel (*24*) in pentane. After flushing with pentane/diethyl ether 9:1 (150 mL), the target compound was isolated with pentane/diethyl ether 7:3 (150 mL). The [$^2H_2$]-δ-decenolactone obtained (yield 30%) was characterized by MS-EI and MS-CI. Because the labeling was introduced in the ring (cf. **Figure 3**), the MS-EI spectrum was dominated by two major fragments: *m*/*z* 99 (100%) and *m*/*z* 70 (90%). In comparison to the MS-EI of the unlabeled δ-decenolactone, in which *m*/*z* 97 (100%) and *m*/*z* 68 (80%) predominated (data not shown), the spectrum of [$^2H_2$]-δ-decenolactone clearly indicated the introduction of two deuterium atoms. This result was confirmed by the MS-CI spectra of the unlabeled and the labeled lactone, respectively, showing base ions at *m*/*z* 169 and 171.

Isolation of the Volatile Fraction. After mixing with water (10 g), followed by 10 min for equilibration, the cocoa powder (50 g) was extracted with diethyl ether (2 × 300 mL) by vigorous stirring at room temperature for 2 h. The extract was filtered and concentrated to ≈150 mL on a Vigreux column (60 × 1 cm), and the volatiles were isolated using the solvent-assisted flavor evaporation (SAFE) method (*33*). The distillate was extracted with an aqueous solution of sodium bicarbonate (0.5 mol/L, 3 × 50 mL) to remove acidic, volatile compounds. The organic phase, containing the neutral−basic volatiles, was washed with aqueous saturated sodium chloride (2 × 50 mL), dried over anhydrous sodium sulfate, and concentrated to 500 μL by distilling off the solvent using a Vigreux column (60 × 1 cm) followed by microdistillation.

The remaining aqueous phase, containing the acidic volatiles, was adjusted to pH 2.5 with hydrochloric acid (2 mol/L) and then extracted with diethyl ether (3 × 100 mL). The combined organic phases were washed with saturated sodium chloride solution (2 × 50 mL), dried over anhydrous sodium sulfate, and concentrated to 500 μL by distilling off the solvent at a Vigreux column (60 × 1 cm), followed by microdistillation.

High-Resolution Gas Chromatography−Olfactometry (HRGC-O). HRGC was performed using a Fisons Instruments 8160 gas chromatograph (Mainz, Germany) and by separating the aroma distillates on the following fused silica capillaries: FFAP (25 m × 0.32 mm, 0.20 μm film thickness; Varian, Darmstadt, Germany), SE-54 (25 m × 0.32 mm, 0.25 μm film thickness; Macherey-Nagel, Düren, Germany); and OV 1701 (30 m × 0.32 mm, 0.25 μm film thickness; Varian). The samples were applied by the cold-on-column injection technique at 40 °C. Helium was used as carrier gas at a flow rate of 2.2 mL/min. After 1 min, the temperature was raised at 40 °C/min to 60 °C (held for 1 min isothermally), then at 6 °C/min to 180 °C, and finally at 15 °C/min to 240 °C (FFAP). Separation on the SE-54 or the OV 1701 stationary phases was done using the following procedure: From 40 to 60 °C at 40 °C/min, after 1 min isothermal, the temperature was raised at 4 °C/min to 140 °C, then at 20 °C/min to 240 °C (held for 5 min isothermally).

For GC-O and AEDA experiments, the effluent was split 1:1 (by volume) at the end of the column via two deactivated fused silica capillaries (40 cm × 0.15 mm i.d.). One part was directed to a flame ionization detector (FID) held at 200 °C and the other part to a sniffing port, held at 180 °C. The neutral−basic and acidic fractions were stepwise diluted with diethyl ether (1:1, v/v) and analyzed by HRGC-O until no odor was detectable at the respective retention index. Odorants detected were assigned to FD factors ≥ 1 (*12*). HRGC-O was performed using aliquots of 0.5 μL, and the linear retention indices (RI) of the compounds were calculated using a series of *n*-alkanes.

High-Resolution Gas Chromatography−Mass Spectrometry (HRGC-MS). For compound identification, mass spectrometric analyses were carried out by means of a Saturn 2000 mass spectrometer (Varian) in tandem with the capillaries described above. Mass spectra were generated in the electron impact mode (MS-EI) at 70 eV and in the chemical ionization mode (MS-CI) at 115 eV using methanol as the reactant gas.

Quantitation by Stable Isotope Dilution Assays (SIDA). Depending on the amount of the respective analyte present, which was estimated

Table 2. Most Odor-Active Compounds (FD ≥ 8) Identified in an Extract from Cocoa Powder

no.	odorant[b]	odor quality[c]	RI[a] on FFAP	RI[a] on SE 54	RI[a] on OV 1701	FD factor[d]
1	methylpropanal	malty	830	–	–	8
2	2[e]- and 3-methylbutanal	malty	920	651	731	1024
3	2-acetyl-1-pyrroline	popcorn-like	1324	927	1019	1024
4	dimethyl trisulfide	sulfury	1332	961	1043	2048
5	2,3,5-trimethylpyrazine	earthy	1394	1011	1081	128
6	2-ethyl-3,6-dimethylpyrazine	earthy	1429	1080	1147	256
7	2-ethyl-3,5-dimethylpyrazine	earthy	1446	1084	1150	2048
8	acetic acid[e]	sour	1448	–	–	512
9	2,3-diethyl-5-methylpyrazine	earthy	1476	1087	1150	256
10	linalool[e]	flowery	1535	1192	–	32
11	methylpropanoic acid[f]	rancid	1558	–	–	64
12	butanoic acid[f]	sweaty	1620	–	–	64
13	phenylacetaldehyde	honey-like	1635	1039	1179	1024
14	2-methyl-3-(methyldithio)furan	cooked meat-like	1655	1165	–	128
15	2[e]- and 3-methylbutanoic acid[f]	sweaty	1660	–	–	4096
16	unknown	sulfury	1721	–	–	32
17	phenylmethyl acetate	fruity, flowery	1748	–	–	32
18	2-phenylethyl acetate	flowery	1804	1382	1474	256
19	2-methoxyphenol	spicy, smoky	1857	1087	1224	512
20	phenylmethanol	flowery	1871	–	–	32
21	2-phenylethanol	flowery	1902	1112	1282	256
22	δ-octalactone[e]	coconut-like	1989	1266	1507	8
23	γ-octenolactone[e]	coconut-like	2019	1260	–	128
24	γ-nonalactone[e]	coconut-like	2024	1365	1540	64
25	4-hydroxy-2,5-dimethyl-3(2*H*)-furanone[f]	caramel-like	2042	–	–	4096
26	4-methylphenol	horse stable, phenolic	2082	1080	1309	64
27	γ-decalactone[e]	coconut-like	2140	1462	–	32
28	δ-decalactone[e]	coconut-like	2184	–	1352	32
29	3-ethylphenol	phenolic	2190	1175	1402	64
30	3-hydroxy-4,5-dimethyl-2(5*H*)-furanone[f]	spicy	2212	–	–	512
31	δ-decenolactone[e]	coconut-like	2238	1477	1724	256
32	*cis*-isoeugenol	smoked	2281	–	1573	128
33	3-methylindole	fecal	2503	–	1626	8
34	2-phenylacetic acid[f]	honey-like	2554	–	–	1024
35	vanillin[f]	vanilla-like	2603	–	–	128

[a] Retention index; –, not determined. [b] The compound was identified by comparing the mass spectra (MS-EI, MS-CI), the retention indices on capillary FFAP, SE-54, and OV 1701, and the odor quality and intensity perceived during sniffing with data obtained for the reference compound. [c] Odor quality perceived at the sniffing port. [d] Flavor dilution factor determined by AEDA on the FFAP capillary. [e] Stereochemistry was not determined. Odor qualities are given for the racemate. [f] Compound was identified in the acidic fraction.

in a preliminary experiment, aliquots of the cocoa powder (from 1 to 100 g, respectively), were suspended in diethyl ether/water (95:5, by vol) and known amounts of the respective labeled isotopologues (**Table 1**), dissolved in diethyl ether, were added. The suspension was stirred for 70 min, and then the volatile fraction was isolated as described above. The SAFE distillate was separated into the neutral−basic and the acidic fractions, and both fractions were analyzed by GC-MS monitoring the intensities of the respective ions given in **Table 1**. In some cases, two-dimensional (TD) GC was applied. TD-HRGC-MS was performed by using the moving column stream switching system (MCSS) (Fisons Instruments, Mainz-Kastell, Germany) as described recently (*34*). The concentrations were calculated from the relative abundances of ions selected for the analyte and the internal standards, and the data were corrected by means of response factors, determined from mixtures containing known concentrations of the labeled and the unlabeled compound (*35*). As indicated in **Table 3**, three different batches of cocoa powder were analyzed.

Sensory Evaluation. Ten assessors with experience in sensory evaluation were recruited from the German Research Center of Food Chemistry. Sensory analyses were performed at 21 ± 1 °C in a sensory panel room, equipped with single booths. Aroma profile analyses were performed by orthonasally scoring 11 odor qualities on a seven-point scale from 0 to 3 (0, 0.5, 1, 1.5, 2, 2.5, 3) selected in a previous session for cocoa powder evaluation. The values given by the panelists were averaged. Orthonasal odor thresholds were determined in sunflower oil by means of the triangle test (*36*).

Aroma Recombination. The matrix for the aroma recombination experiments was obtained in the following way: A mixture of cocoa powder (100 g) and water (10 mL) was exhaustively extracted with diethyl ether (3 × 500 mL). The residue was suspended in 300 mL of water and freeze-dried. By repeating the extraction/freeze-drying, an odorless powder was obtained. The following mixture of 24 aroma compounds was prepared in sunflower oil (50 mL): 3-methylbutanal (1280 μg); 2-methylbutanal (715 μg); 3-methylbutanoic acid (430 μg); phenylacetaldehyde (330 μg); phenylacetic acid (385 μg); methylpropanoic acid (140 μg); 2-methylbutanoic acid (88 μg); 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (31 μg); 2-phenylethanol (30 μg); butanoic acid (16 μg); phenylethyl acetate (16 μg); 2-methoxyphenol (12 μg); 4-methylphenol (6.2 μg); linalool (3.6 μg); 3-methylindole (2.7 μg); 2-ethyl-3,6-dimethylpyrazine (3.5 μg); 2-ethyl-3,5-dimethylpyrazine (1.5 μg); 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (0.72 μg); 2,3-diethyl-5-methylpyrazine (0.40 μg); dimethyl trisulfide (0.35 μg); 2-acetyl-1-pyrroline (0.30 μg); 2-methyl-3-(methyldithio)furan (0.024 μg); 2-isobutyl-3-methoxypyrazine (0.0015 μg). This mixture and acetic acid (16.5 mg) were dissolved in 12.5 g of deodorized cocoa butter and mixed with 37.5 g of the defatted, deodorized cocoa powder described above. The recombinate was frozen with liquid nitrogen and then ground in a laboratory mill (A10, Jahnke & Kunkel, Staufen, Germany).

RESULTS AND DISCUSSION

Identification of Odor-Active Constituents. For GC-O, the volatile fraction from cocoa powder was isolated by solvent extraction followed by high-vacuum distillation to remove the nonvolatile material. The aroma of this distillate fully represented the characteristic odor of cocoa when a drop of the ethereal solution was vaporized on a strip of filter paper.

Table 3. Concentrations of 30 Odor-Active Compounds in the Cocoa Powder

odorant	concn (μg/kg) mean	SD[a]
acetic acid	332000	9650
3-methylbutanal	25770	2270
2-methylbutanal[b]	14314	529
3-methylbutanoic acid	8550	430
2-phenylacetic acid	7700	399
phenylacetaldehyde	6600	157
methylpropanoic acid	2800	57
2-methylbutanoic acid[b]	1750	88
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	620	41
2-phenylethanol	590	22
butanoic acid	320	13
2-phenylethyl acetate	315	6.0
2-methoxyphenol	230	26
2,3,5-trimethylpyrazine	200	7.5
δ-decenolactone[b]	185	10
δ-octenolactone[b]	151	4.2
4-methylphenol	124	5.7
δ-decalactone[b]	82	2.0
linalool[b]	72	5.7
2-ethyl-3,6-dimethylpyrazine	70	3.6
γ-decalactone[b]	60	0.65
3-methylindole	55	2.1
γ-nonalactone[b]	43	2.0
2-ethyl-3,5-dimethylpyrazine	31	1.1
3-hydroxy-4,5-dimethyl-2(5*H*)-furanone	15	0.037
γ-octalactone[b]	14	0.43
2,3-diethyl-5-methylpyrazine	8.2	0.50
dimethyl trisulfide	6.9	0.035
2-acetyl-1-pyrroline	5.9	0.11
2-methyl-3-(methyldithio)furan	0.47	0.013
2-isobutyl-3-methoxypyrazine	0.85	0.042

[a] Standard deviation calculated from quantitative data obtained from at least three different batches. [b] Stereoisomers, if present, were not separated.

Table 4. Odor Thresholds and Odor Activity Values (OAVs) of Important Aroma Compounds in Cocoa Powder

odorant	odor threshold[a] (μg/kg)	OAV[b]
acetic acid	124[c]	2680
3-methylbutanal	13[c]	1980
3-methylbutanoic acid	22[c]	390
phenylacetaldehyde	22[c]	300
2-methylbutanal	140[c]	102
3-hydroxy-4,5-dimethyl-2(5*H*)-furanone	0.2[c]	75
2-acetyl-1-pyrroline	0.1[c]	59
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	25[c]	25
2-phenylacetic acid	360[d]	21
2,3-diethyl-5-methylpyrazine	0.5[c]	16
methylpropanoic acid	190	15
2-ethyl-3,5-dimethylpyrazine	2.2[c]	14
2-methoxyphenol	16[c]	14
2-isobutyl-3-methoxypyrazine	0.8[c]	12
2-methylbutanoic acid	203[d]	8.7
3-methylindole	16[c]	3.5
2-phenylethanol	211[c]	2.8
dimethyl trisulfide	2.5[c]	2.8
butanoic acid	135[c]	2.4
linalool	37	2.0
4-methylphenol	68	1.8
2-phenylethyl acetate	233[d]	1.3
2-ethyl-3,6-dimethylpyrazine	57[c]	1.2
2-methyl-3-(methyldithio)furan	0.4[c]	1.2
δ-octenolactone	4730[d]	<1
δ-octalactone	2490[d]	<1
δ-decenolactone	590	<1
δ-decalactone	400[c]	<1
γ-decalactone	320[c]	<1
2,3,5-trimethylpyrazine	290	<1
γ-nonalactone	148[c]	<1

[a] Orthonasal odor thresholds determined in sunflower oil. [b] OAVs were calculated by dividing the concentration of an odorant by its orthonasal detection threshold. [c] Threshold values according to ref *37*. [d] R. Schmitt and P. Schieberle (unpublished results).

Application of HRGC-O on an aliquot of the aroma distillate revealed 27 aroma-active regions in the neutral−basic fraction (**Figure 4**) and 8 additional areas in the acidic fraction (data not shown). By sniffing of serial dilutions of the extract containing the neutral−basic volatiles (1:1), compounds **4** and **7**, exhibiting a sulfury and an earthy, potato-like aroma, respectively, showed the highest FD factors of 2048, followed by compounds **2** (malty), **3** (roasty, popcorn), and **13** (honey-like). An AEDA of the fraction containing the acidic volatile compounds (data not shown) revealed 3 additional compounds with high FD factors of ≥1024, eliciting sweaty (**15**; **Table 2**), caramel-like (**25**), and sweet, honey-like aromas (**34**).

To obtain enough material for the identification experiments, the volatiles from 500 g of cocoa powder were isolated and separated into the neutral−basic and acidic fractions. Interestingly, both fractions no longer represented the overall cocoa aroma, but the odor was regained when both fractions were recombined after separation.

First, the acidic fraction was separated on the FFAP column, and mass spectra were recorded while a panelist was sniffing the eluate in parallel. The mass spectrum monitored at the most intense odor was compared with data of an in-house database containing >700 spectra of key food odorants. On the basis of the proposed structure, the respective reference compound was analyzed and the odor quality and intensity were compared with the sensory perception (odor quality and intensity) at the same retention index as detected in the cocoa extract. This procedure avoids incorrect identifications, if the characterization were to be based on only mass spectra and/or retention indices, because aroma attributes can, thus, be used as a further criterion for compound identification. The most odor-active compounds in the acidic fraction were identified as 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (**25**; **Table 2**), 2- and 3-methylbutanoic acid (**15**), and 2-phenylacetic acid (**34**). Somewhat lower FD factors were determined for 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (**30**) and acetic acid (**8**).

Application of the same identification procedure on the neutral−basic extract containing 27 odor-active compounds in the FD factor range of 8−4096 allowed the identification of all constituents except compound **16**, showing a quite low FD factor. Compounds with the highest FD factors were identified as dimethyl trisulfide (**4**; **Table 2**), 2-ethyl-3,5-dimethylpyrazine (**7**), and phenylacetaldehyde (**13**) followed by 2- and 3-methylbutanal (**2**) and 2-acetyl-1-pyrroline (**3**).

The results (**Table 2**) confirmed an important role of Strecker aldehydes and pyrazines in the aroma of the cocoa powder, but were quite different from the data previously obtained by us for a commercial cocoa mass (*11*). Although, in particular, esters, such as ethyl 3-methylbutanoate, were lacking in the cocoa powder, other odorants, such as 2-acetyl-1-pyrroline or dimethyl trisulfide, appeared with much higher FD factors in the cocoa powder than in the cocoa mass. However, it must be taken into consideration that the cocoa mass previously analyzed was "natural" and had not been treated with alkali (dutching). Among the odorants, in particular, *cis*-isoeugenol is reported here for the first time in cocoa.

Quantitative Analysis. Quantitative data are a prerequisite to elucidate the importance of odor-active compounds to the food itself. The most reliable quantitative data are obtained by

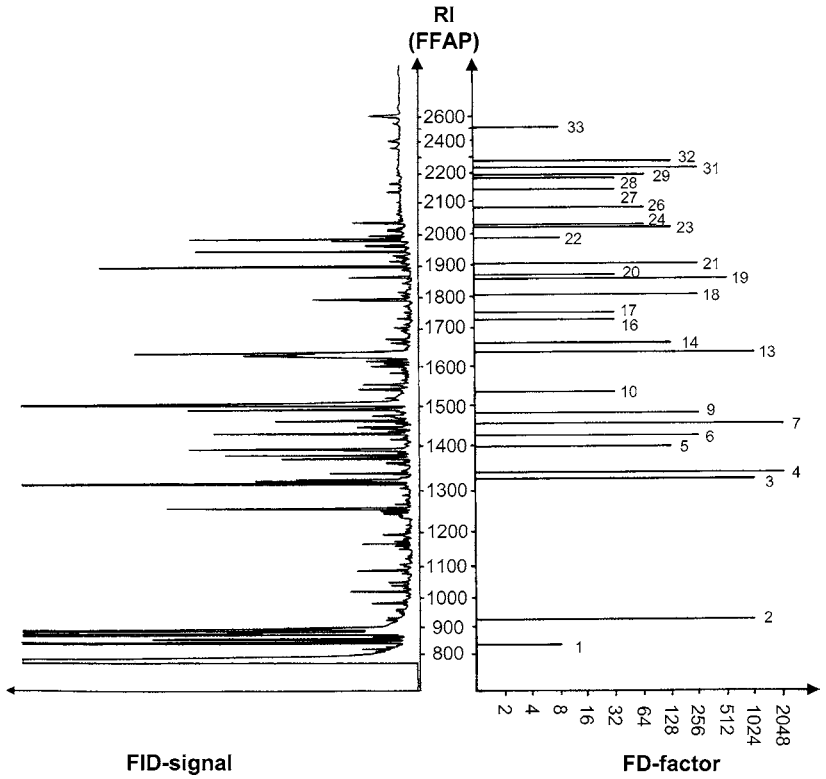

Figure 4. Gas chromatogram (left) of the neutral-basic volatiles isolated from cocoa powder and flavor dilution chromatogram (right) obtained by drawing the FD factors over the linear retention indices. Numbering refers to **Table 2**.

SIDAs using an isotopology of the respective aroma compound as the internal standard (*12*). However, most of these compounds are not commercially available and, thus, have to be synthesized. In **Figure 1**, the structures of 26 isotopologies used in the isotope dilution assays are shown indicating the positions labeled by ≈100% with deuterium or carbon-13, respectively. Using the different molecular ions recorded by MS-CI for the odorant and the internal standard, the concentrations of the analyte could be exactly measured without a determination of time-consuming recovery rates.

The results of the quantitative experiments are summarized in **Table 3**. Being present in a concentration of 332 mg/kg, acetic acid was by far the most abundant odorant, followed by considerable amounts of 3-methylbutanal (25.7 mg/kg) and 2-methybutanal (14.3 mg/kg). Slightly lower concentrations were found for 3-methylbutanoic acid, phenylacetic acid, phenylacetaldehyde, methylpropanoic acid, and 2-methylbutanoic acid. On the other side of the scale, dimethyl trisulfide, 2-acetyl-1-pyrroline, 2-isopropyl-3-methoxypyrazine, and 2-methyl-3-(dithio)furan were determined in very low amounts.

Although always the same brand was used in this study, it might be assumed that the concentrations of the aroma compounds may vary depending on the single batches analyzed. As indicated in **Table 3**, up to four different batches were analyzed. The results revealed quite low batch-to-batch differences in the range of ≈5−10%.

Calculation of Odor Activity Values (OAVs). To estimate whether the odorants quantified are present above their odor thresholds in the cocoa powder, the OAVs (ratio of concentration to odor threshold) were calculated. Most of the odor thresholds used had previously been determined in our group (*37*), but data for 3-methylphenol, δ-decenolactone, methylpropanoic acid, and 2,3,5-trimethylpyrazine had to be completed. Among the odorants considered, 2-acetyl-1-pyrrroline, 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone, 2-methyl-3-(methyldithio)furan, and 2-isobutyl-3-methoxypyrazine showed the lowest odor threshold in sunflower oil (**Table 4**).

A calculation of the OAVs revealed the highest values for acetic acid and 3-methylbutanal and a somewhat lower "odor activities" for 3-methylbutanoic acid, phenylacetaldehyde, and 2-methylbutanal. In total, 24 of the compounds investigated showed a concentration in cocoa powder above the respective threshold, respectively an OAV > 1, and are, therefore, suggested to be relevant for the aroma of cocoa powder. Interestingly, none of the six lactones exceeded their odor threshold, and also 2,3,5-trimethylpyrazine was present below its odor threshold.

Aroma Recombination Experiments. OAVs indicate whether a single compound is present above a threshold in a given matrix and should, therefore, contribute to a given aroma. However, the application of the OAV concept is not able to indicate how interactions of single key odorants showing a broad range of different odor qualities can finally lead to the overall aroma of the food itself. This, however, can be addressed by aroma recombination experiments (*12*).

Therefore, to confirm the selection of odorants made by using AEDA and the quantitative measurements, an aroma recombination based on the quantitative results was performed. For this purpose, a mixture containing all 24 odorants with OAVs > 1 (**Table 4**) was made up in sunflower oil using the same concentrations as these were determined in the cocoa powder. This mixture was added to deodorized cocoa butter and mixed with deodorized, defatted cocoa powder. The aroma of the model was evaluated by a sensory panel in comparison with the cocoa powder. The results presented as a spider web diagram (**Figure 5**) showed a good similarity of the aroma of the model and the overall aroma of the cocoa powder. Although the intensities of the odor qualities honey-like, caramel-like, rancid−sweaty, and, in particular, malty were rated slightly higher in the model than in cocoa powder, the overall aroma of the recombinate was

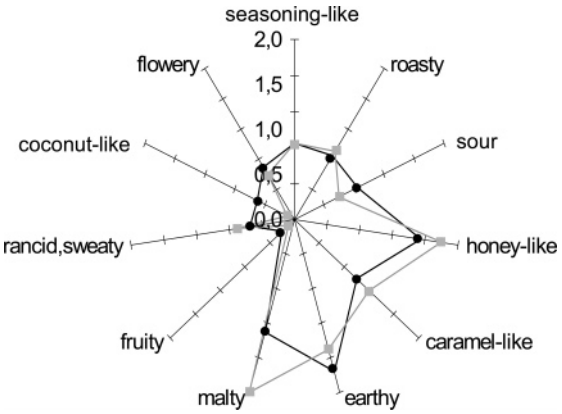

Figure 5. Orthonasal flavor profiles of the cocoa powder (gray squares) and the aroma model (●).

unanimously perceived as typically cocoa-like, whereas a blank model (deodorized, defatted cocoa powder plus deodorized cocoa butter) without the aroma mixture was nearly odorless.

The results clearly indicate that the "sensomics" concept is a valuable tool to define food aromas on the basis of the key aroma compounds present. Because no individual compound was identified bearing the typical aroma of cocoa, in particular the aroma recombination is a crucial step to overcome the present lack in knowledge on the interactions of single aroma compounds when stimulating the human odorant receptors in the olfactory bulb during aroma perception. The data are also a reliable basis to control and improve the aroma of cocoa products by quantitatively assessing the key aroma compounds suggested.

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