Quantification of Key Odorants Formed by Autoxidation of Arachidonic Acid Using Isotope Dilution Assay

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ABSTRACT: Six odor-active compounds generated by autoxidation of arachidonic acid (AA) were quantified by isotope dilution assay (IDA), i.e., hexanal (**1**), 1-octen-3-one (**2**), (*E,Z*)-2,4-decadienal (**3**), (*E,E*)-2,4-decadienal (**4**), *trans*-4,5-epoxy-(*E*)-2-decenal (**5**), and (*E,Z,Z*)-2,4,7-tridecatrienal (**6**). Compound **1** was the most abundant odorant with about 700 mg/100 g autoxidized AA, which corresponds to 2.2 mol% yield. Based on the odor activity values (ratio of concentration to odor threshold), odorants **3** (fatty) and **5** (metallic) showed the highest sensory contribution followed by **1** (green), **2** (mushroom-like), **6** (egg white-like), and **4** (fatty). For the first time, reliable quantitative results are reported for odorants **1–6** in autoxidized AA, in particular odorant **6**, which is a characteristic compound found in autoxidized AA. Synthesis of deuterated **6**, required for IDA, is described in detail. The formation of odorants **1–6** by autoxidation of AA is discussed with respect to the quantitative data.

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Arachidonic acid (AA, 20:4) is found in membrane phospholipids of all mammalian tissues and plays a role in the regulation of functional properties like fluidity, permeability, and activity of membrane-bound enzymes. Although it is not an essential fatty acid, AA has recently been shown to correlate with both intrauterine growth of preterm infants (1,2) and growth in the first year of life (3). There is an increasing interest in the production of highly purified oils rich in AA for infant formulas (4).

As a polyunsaturated fatty acid, AA is very susceptible to oxidation with atmospheric oxygen and thus brings about losses in nutritional quality. Oxidative degradation also gives rise to flavor deterioration, i.e., formation of stale and rancid off-flavors. Off-flavor caused by lipid oxidation has been the subject of numerous studies, mainly on unsaturated lipids containing oleic, linoleic, and linolenic acids (5,6). However, little work has been published on volatile compounds formed by autoxidation of AA or methyl arachidonate. Depending on the starting material, reaction conditions, and analytical techniques used, various volatile compounds were identified, such as hexanal (7–10), 2-heptenal (7–9), 2,4-decadienal (8,10), 2,4,7-tridecatrienal (8), 1-octen-3-ol (9), 1-octen-3-one (9), pentane (10), and methyl 5-oxopentanoate (10).

The volatile composition of autoxidized AA has recently been characterized with focus on odor-active constituents (11,12). Hexanal (**1**), 1-octen-3-one (**2**), (*E,Z*)-2,4-decadienal (**3**), (*E,E*)-2,4-decadienal (**4**), *trans*-4,5-epoxy-(*E*)-2-decenal (**5**), and (*E,Z,Z*)-2,4,7-tridecatrienal (**6**) revealed high sensory relevance among the 19 odorants detected by gas chromatography–olfactometry (GC–O). The aroma properties of the most potent odorants were in good agreement with the overall aroma of autoxidized AA, described as green, metallic, egg white-like, fatty, and fishy.

It is well known that results obtained by GC–O only roughly estimate the sensory relevance of odorants, mainly because of discrimination during isolation of volatiles. The isotope dilution assay (IDA) has been shown to be a sensitive, accurate, and reliable quantification technique in flavor research (13–15). This method involves spiking food materials with known amounts of a labeled substance prior to sample preparation and analysis by gas chromatography–mass spectrometry (GC–MS). In this way, losses can be accounted for because whatever changes occur in the natural substance also occur in the labeled version. IDA has also been applied to lipid degradation products and off-flavor studies (14,16,17).

In this paper we report quantitative data of key odorants generated by autoxidation of AA, using the IDA technique for five aldehydes and one ketone. Synthesis of fourfold deuterated **6** is also described.

EXPERIMENTAL PROCEDURES

Materials. The following chemicals were obtained commercially: AA (99%), ethylmagnesium bromide, palladium on $CaCO_3$ (Lindlar's catalyst) (Aldrich, Buchs, Switzerland); deuterium gas (Carbagas, Lausanne, Switzerland); neutral aluminum oxide (Al_2O_3), hexanal, cuprous chloride (CuCl), manganese (IV) oxide (MnO_2), phosphorus tribromide (PBr_3), pyridine (H_2O <0.005%), tetrahydrofuran (H_2O <0.005%) (Fluka, Buchs, Switzerland); 2,4-decadienal (*E,E*: 95%; *E,Z*: 5%; Fontarom, Cergy Pontoise, France); deuterated chloroform (C^2HCl_3, 99.8%, Dr. Glaser AG, Basel, Switzerland); diethyl ether (Et_2O), hexane, pentane, silica gel

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Abbreviations: AA, arachidonic acid; CC, column chromatography; CI, chemical ionization; EI, electron ionization; GC, gas chromatography; GC–O, gas chromatography–olfactometry; HPET, hydroperoxyeicosatrienoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HPOD, hydroperoxyoctadecadienoic acid; IDA, isotope dilution assay; IS, internal standard; MS, mass spectrometry; NMR, nuclear magnetic resonance; OAV, odor activity value; PCI, positive chemical ionization; RI, retention index; SIM, selected ion monitoring.

60, sodium carbonate (Na_2CO_3), sodium hydrogen carbonate ($NaHCO_3$), anhydrous sodium sulfate (Na_2SO_4), sulfuric acid (H_2SO_4) (Merck, Darmstadt, Germany); 2-octyn-1-ol, (*E*)-2-penten-4-yn-1-ol (Lancaster, Morecambe, England); and 1-octen-3-one (Oxford, Brackley, United Kingdom).

Silica gel 60 and neutral aluminum oxide were treated as reported by Ullrich and Grosch (18). *trans*-4,5-Epoxy-(*E*)-2-decenal (**5**) and (*E,Z,Z*)-2,4,7-tridecatrienal (**6**) were synthesized as described earlier (12,19). The labeled internal standards [5,6-$^2H_2$]-hexanal (***d*-1**), [1-$^2H_{1;2}$,2-$^2H_{1;1}$]-1-octen-3-one (***d*-2**), [3,4-$^2H_2$]-(*E,E*)-2,4-decadienal (***d*-4**), and [4,5-$^2H_2$]-*trans*-4,5-epoxy-(*E*)-2-decenal (***d*-5**) were synthesized as previously reported (19–21).

Autoxidation. AA (500 mg) was dissolved in freshly distilled Et_2O (50 mL) and placed into a 250-mL flask. The solvent was removed with a stream of nitrogen to obtain a thin layer of the lipid material. The flask was filled with oxygen and sealed. After storing the sample in the dark for 48 h at room temperature, the flask was flushed with nitrogen (1 min). The peroxide value was measured using the Fe test (22), indicating the presence of about 60 mol% of total peroxides.

Clean-up. Nonvolatile components were removed by column chromatography (CC) for quantitative analysis. Et_2O (2 mL) was added to the reaction mixture after autoxidation. The solution was spiked with labeled internal standards dissolved in pentane. The homogenized mixture was applied onto a glass column (20 × 1 cm) packed with Al_2O_3 in pentane/Et_2O (2:1, vol/vol). The column was maintained at 10°C by a cooling jacket. Compounds without a carboxylic group were eluted with 150 mL pentane/Et_2O (2:1, vol/vol). The effluent was collected and concentrated to 2 mL using a Vigreux column (50 × 1 cm).

In order to further purify the samples for quantification, the concentrated effluent obtained from CC with Al_2O_3 was applied to a glass column (20 × 1 cm, with cooling jacket) packed with silica gel 60 in pentane. Elution was performed stepwise with 50 mL pentane/Et_2O (98:2, vol/vol) and 150 mL pentane/Et_2O (85:15, vol/vol). Only the second fraction was collected and concentrated to 0.5 mL for GC–MS analysis.

Capillary GC. This was performed with a Carlo Erba Mega 2 (Fisons Instruments, *via* Brechbühler, Schlieren, Switzerland) equipped with a cold on-column injector and a flame-ionization detector held at 230°C. Fused-silica capillary columns of low (DB-5), medium (DB-1701), and high (DB-Wax, DB-FFAP) polarity were used (J&W Scientific MSP Friedli, Koeniz, Switzerland), both 30 m × 0.32 mm with a film thickness of 0.25 μm. Helium (80 kPa) was used as carrier gas. The temperature program was 35°C (2 min), 40°C/min to 50°C (1 min), 6°C/min to 180°C, 10°C/min to 240°C (10 min). Linear retention indices (RI) were calculated according to van den Dool and Kratz (23).

GC–MS. Qualitative analysis was performed on a MAT 8430 mass spectrometer (Finnigan, Bremen, Germany). Electron ionization (EI) mass spectra were generated at 70 eV. Chemical ionization (MS–CI) was performed at 150 eV with ammonia as the reagent gas. Further details of the GC–MS system and chromatographic conditions were described elsewhere (20). Relative abundances of the ions are given in percentages.

Quantitative analysis was performed on a Finnigan SSQ 7000 mass spectrometer coupled with an HP-5890 gas chromatograph. CI was carried out at 200 eV with isobutane as reagent gas. Samples were introduced *via* splitless injection at 250°C on a DB-1701 capillary column (30 m × 0.32 mm, film thickness 0.25 μm; J&W Scientific). Helium (90 kPa) was used as carrier gas. Temperature program: 20°C (1 min), 70°C/min to 60°C, 6°C/min to 180°C, 10°C/min to 240°C (10 min). Each sample (2 μL) was injected at least twice. Quantitative measurements were carried out in full scan or, if necessary, in the selected ion monitoring (SIM) mode measuring characteristic ions listed in Table 1.

Determination of isotopic purity. This was calculated from GC–MS data as recently described for deuterated aldehydes (20). Clusters of ions representing the species from $[M + 3]^+$ to $[M - 2]^+$ of both the deuterated standard and nonlabeled reference compound were measured in the positive chemical ionization (PCI) mode on the SSQ 7000 using SIM and isobutane as reagent gas (24,25). The nondeuterated substance was analyzed for isotope correction of the labeled compound. The deuterium distribution was calculated according to Rohwedder (25).

TABLE 1
Parameters Used in the Quantification of Six Lipid-Derived Odorants by Isotope Dilution Assay

Analyte	Internal standard (IS)	Selected ions (*m/z*)[a]		Linearity[b]	Linear range[b]
		Analyte	IS	(r^2)	(ratio analyte/IS)
1	[5,6-$^2H_2$]-Hexanal (***d*-1**)	101	103	0.999	0.05–19.0
2	[1-$^2H_{1;2}$,2-$^2H_{1;1}$]-1-Octen-3-one (***d*-2**)	127	129/130[c]	0.999	0.05–9.0
3	[3,4-$^2H_2$]-(*E,E*)-2,4-Decadienal (***d*-4**)	153	155	0.999	0.05–9.0
4	[3,4-$^2H_2$]-(*E,E*)-2,4-Decadienal (***d*-4**)	153	155	0.999	0.05–9.0
5	[4,5-$^2H_2$]-*trans*-4,5-Epoxy-(*E*)-2-decenal (***d*-5**)	153	155	0.999	0.05–9.0
6	[4,5,7,8-$^2H_4$]-(*E,Z,Z*)-2,4,7-Tridecatrienal (***d*-6**)	193	197	0.999	0.05–9.0

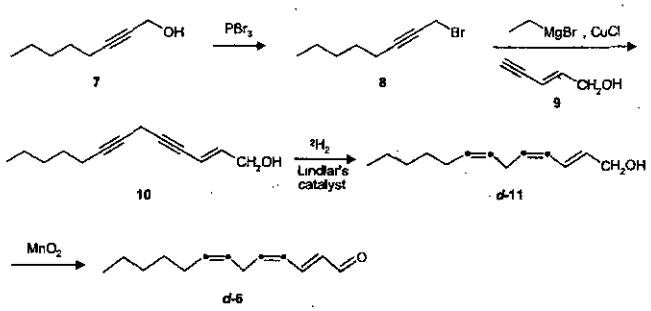
[a]Chemical ionization was applied using isobutane as reagent gas. The ion pairs measured generally were the species $[M + H]^+$, except for **5** and ***d*-5** where $[M + H - O]^+$ was monitored.
[b]Linearity and linear range were obtained from the calibration graphs using selected ions (see the Experimental Procedures section).
[c]***d*-2** was composed of two major isotopomers (21). The sum of both ions (*m/z* 129 and 130) was used for establishing the calibration graph.

IDA. Defined amounts of labeled internal standard (IS) in solution were added to autoxidized AA before isolation of volatiles by CC. Calibration curves were obtained using mixtures of defined amounts of analyte and labeled IS (14). As recently described for **5** and ***d*-5** (19), nine mixtures **1**/***d*-1** were used, i.e., from 0.5 + 9.5, 1 + 9, 2 + 8, 3 + 7, and 5 + 5 to 7 + 3, 8 + 2, 9 + 1, and 9.5 + 0.5. Calibration curves were established for the odorants **1**, **2**, **4**, **5**, and **6**, while (*E,Z*)-2,4-decadienal (**3**) was quantified using that of **4**. The parameters used in the IDA of the five odorants are summarized in Table 1. Samples for establishing the calibration curves and for quantification were injected twice.

Nuclear magnetic resonance (NMR) spectroscopy. The sample for NMR spectroscopy was prepared in a WILMAD 528-PP 5 mm Pyrex NMR tube (Textronica AG, Oberreit, Switzerland), using as solvent C^2HCl_3 (0.7 mL) from a sealed vial. The NMR spectra were acquired on a Bruker AM-360 spectrometer, equipped with a quadrinuclear 5-mm probe head, at 360.13 MHz for ^1H and at 90.56 MHz for ^{13}C under standard conditions (20). All shifts are cited in ppm from the internal trimethylsilane standard. Since nondeuterated (*E,Z,Z*)-2,4,7-tridecatrienal had been previously elucidated in detail (12), one-dimensional spectra (^1H NMR, ^{13}C NMR, proton decoupled and nondecoupled, distortionless enhancement by polarization transfer 135) were sufficient to characterize its deuterated analog.

*Synthesis of [4,5,7,8-$^2H_4$]-(E,Z,Z)-2,4,7-tridecatrienal (**d-6**).* The synthesis procedure used for (*E,Z,Z*)-2,4,7-tridecatrienal (12) was adapted to obtain ***d*-6** by partial deuteration of intermediate **10** as shown in Scheme 1. Experimental details for the first two steps leading to **10** are described elsewhere (12).

*[4,5,7,8-$^2H_4$]-(E,Z,Z)-2,4,7-Tridecatrien-1-ol (**d-11**).* This was prepared by partial deuteration of **10** in CH_3O^2H at room temperature under normal pressure with Lindlar's catalyst following the conditions recently described (12). Monitoring of the reaction by GC–MS indicated ***d*-11** as the major compound with some under- and over-deuterated by-products. The product mixture was used in the next step without purification. MS(EI) *m/z* (% relative abundance): 82 (100), 83 (66), 81 (60), 97 (58), 96 (46), 167 (45), 70 (42), 69 (41), 95 (38), 84 (37), 57 (36), 85 (35), 79 (35), 56 (35), 71 (34), 80 (33), 55 (27), 198 (25, M^+), 110 (21), 109 (21), 108 (19), 122 (17), 123 (15), 138 (8), 137 (8), 155 (5). MS(CI), ammonia *m/z* (% relative abundance): 181 (100, $[M + H - H_2O]^+$), 198 (20, M^+ or $[M + NH_4 - H_2O]^+$), 216 (10, $[M + NH_4]^+$).



SCHEME 1

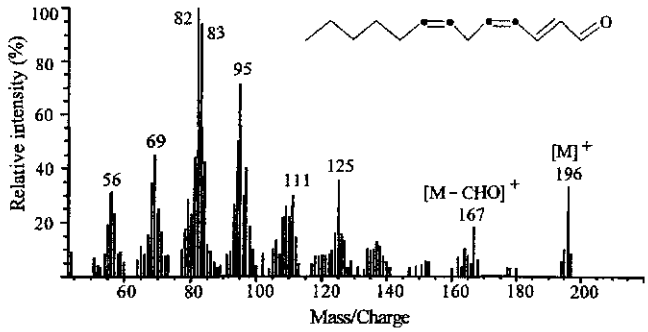


FIG. 1. Mass spectrum (electron ionization) of [4,5,7,8-$^2H_4$]-(*E,Z,Z*)-2,4,7-tridecatrienal (***d*-6**).

*[4,5,7,8-$^2H_4$]-(E,Z,Z)-2,4,7-Tridecatrienal (**d-6**).* The product mixture (1.0 g) containing ***d*-11** obtained in the previous step was oxidized with MnO_2 (10.0 g) in hexane. The oxidation, monitored by GC, was complete after 30 min. The target compound ***d*-6** was obtained with at least 80% (GC) purity by CC. GC: RI (DB-5) = 1581, RI (OV-1701) = 1731, RI (FFAP) = 2118, RI (DB-Wax) = 2105. MS(EI) of ***d*-6** is shown in Figure 1. MS(CI), ammonia *m/z* (% relative abundance): 197 (100, $[M + H]^+$), 214 (80, $[M + NH_4]^+$). The ^1H and ^{13}C NMR spectral data of ***d*-6** are given in Table 2.

RESULTS AND DISCUSSION

*Synthesis of [4,5,7,8-$^2H_4$]-(E,Z,Z)-2,4,7-Tridecatrienal (**d-6**).* The IS ***d*-6** was synthesized in four steps (Scheme 1) in analogy to the nondeuterated compound **6** (12). Bromination of 2-octyn-1-ol (**7**) followed by coupling of the resulting 1-bromo-2-octyne (**8**) with the Grignard derivative of (*E*)-2-penten-4-yn-1-ol (**9**) gave rise to (*E*)-2-tridecen-4,7-diyn-1-ol (**10**). Partial deuteration of **10** using Lindlar's catalyst was employed to obtain all-*cis* configuration for the C4-C5 and C7-C8 double bonds in the resulting [4,5,7,8-$^2H_4$]-(*E,Z,Z*)-2,4,7-tridecatrien-1-ol (***d*-11**). The mild and neutral oxidant MnO_2 was used to oxidize the allylic alcohol ***d*-11** to the target compound (***d*-6**) without changing the *cis* configuration of the C4-C5 double bond, which is sensitive to acidity.

The MS–CI data *m/z* 197, 214 of ***d*-6** compared to *m/z* 193, 210 of **6** (12) indicated incorporation of four deuterium atoms in ***d*-6**. Ions *m/z* 197 and 214 represent the species $[M + H]^+$ and $[M + NH_4]^+$, respectively. The fragments *m/z* 196, 167, and 125 in the EI mass spectrum (Fig. 1) confirmed the presence of four deuterium atoms as compared to *m/z* 192, 163, and 121 in **6** (12).

Signals of the ^1H and ^{13}C NMR spectral data of ***d*-6** (Table 2) were assigned by comparison with the spectral data of **6** (12). The proton spectrum of ***d*-6** was almost identical to that of **6**, when the changes due to the deuteration were taken into account. The spectrum indicated approximately 95% deuterium substitution at each of the expected positions C4, C5, C7, and C8, and also that either two deuterium atoms or one

TABLE 2
^{1}H and ^{13}C NMR Data of [4,5,7,8-$^2H_4$]-(*E,Z,Z*)-2,4,7-Tridecatrienal (*d*-6) in C^2HCl_3[a]

Group	^{1}H NMR[b]	^{13}C NMR[c]
1-CHO	9.62, *d*, 1 H, $J_{1,2}$ = 8.0 Hz	193.9, *d*
2-CH	6.17, *d d*, ≤ 1 H, $J_{2,3}$ = 15.3 Hz, $J_{1,2}$ = 8.0 Hz	132.0, *d*
3-CH	7.476, *d* (~1:1:1), ~0.95 H, $J_{2,3}$ = 15.2 Hz, $^3J_{3,4\text{-}^2H}$ ~1.6 Hz, and 7.479, *d d*, ~0.05 H[d], $J_{2,3}$ = 15.2 Hz, $J_{3,4}$ = 11.5 Hz	146.5, *d*
4-CH	6.27, *d, sl. br.*, ~0.05H[e], $J_{3,4}$ ~11.3 Hz	126.4, *d*[f]
4-C^{2}H	—	126.1, *s* (1:1:1), $^1J_{C^2H}$ = 24.2 Hz
5-CH	5.96, *"t", sl. br.*, ~0.06 H[e], $J_{5,6}$ ~7.6 Hz	141.5, *d*[f]
5-C^{2}H	—	141.1, *s* (1:1:1), $^1J_{C^2H}$ = 24.0 Hz
6-CH$_2$	3.08, *s, sl. br.*, ~1.92 H[g]	26.5, *t*
7-CH	5.35, *t* "quintet," *sl. br.*, ~0.04 H[e], $J_{6,7}$ = 7.2 Hz, $J_{\text{long range avg.}}$ ~1.4 Hz	125.1, *d*[f]
7-C^{2}H	—	124.8, *s* (1:1:1), $^1J_{C^2H}$ = 24.0 Hz
8-CH	5.50, *t* "quintet," *sl. br.*, ~0.04 H[e], $J_{8,9}$ = 7.3 Hz, $J_{\text{long range avg.}}$ ~1.5 Hz	132.0, *d*[f]
8-C^{2}H	—	131.7, *s* (1:1:1), $^1J_{C^2H}$ = 23.4 Hz
9-CH$_2$	2.08, *t, sl. br.*, ≥2 H, $J_{avg.}$ = 7.2 Hz	27.2, *t*
10-CH$_2$	1.38, *m*, ≥2 H, $J_{avg.}$ ~6.9 Hz	29.2, *t*
11-CH$_2$	1.36–1.24, *m*, ≥4 H[h]	31.5, *t*
12-CH$_2$		22.6, *t*
13-CH$_3$	0.89, *t*, ≥3 H, $J_{12,13}$ ~6.9 Hz	14.1, *q*

[a]Chemical shifts (δ) in ppm from internal trimethylsilane.
[b]^{1}H nuclear magnetic resonance (NMR) multiplicity abbreviations: *s* = singlet, *d* = doublet, *t* = triplet, *q* = quartet, *m* = multiplet, *d t* = doublet of triplets (with decreasing values of coupling constants). Quotes ("...") mean approximate description of the multiplet, *sl. br.* = slightly broadened by unresolved long-range couplings. The coupling constants *J* are directly extracted from the spectrum, after moderate Gaussian resolution enhancement, without equalizing the constants belonging to the respective coupling partners. The coupling partners are indicated by subscripts where there is no ambiguity. Since the T_1 relaxation times may be long in this richly deuterated molecule, minor integration errors are not excluded.
[c]^{13}C NMR multiplicity abbreviations: *s, d, t, q*, denominate quaternary, CH-, CH$_2$- and CH$_3$-carbons, respectively.
[d]Signal due to isotopomer(s) not deuterated at 4-C.
[e]Residual proton signals of threefold (or less) deuterated isotopomers.
[f]Due to residual protonated carbons.
[g]Dominant signal, accompanied by several small slightly broad singlet signals between 3.1 and 2.9 ppm, likely due to threefold (or less) deuterated isotopomers and/or double-bond stereoisomers.
[h]The proton signals of 11-CH$_2$ and 12-CH$_2$ are overlapping; see (12) for a more precise shift determination from a two-dimensional heteronuclear correlation experiment in the nondeuterated analog.

deuterium and one proton are found on a double bond, while the doubly protonated double bonds are negligible. The configuration of the double bonds was deduced from that of **6**. There, coupling constants of *ca.* 15.3, 10.6, and 10.6 Hz had been found for the C_2-C_3, C_3-C_5, and C_7-C_8 double bonds, respectively (12). This, together with the nuclear Overhauser effect results, had clearly indicated the *E,Z,Z* configuration.

For an incompletely deuterated substance, integration is complicated by the various isotopomers that occur. The 3-H signal of ***d*-6**, for example, was a superposition of two components. The dominating component (~95% of the integral area) was a doublet (due to the coupling to 2-H) of approximate 1:1:1 patterns (the latter caused by the coupling to the quadrupolar deuterium atom replacing 4-H). This signal component represented four different isotopomers, since the majority of the molecules was at least threefold deuterated. The minor 3-H signal component (~5% integral area) was a doublet of doublets without deuterium coupling, standing for one isotopomer with 4-H instead of 4-^{2}H. The respective integral areas were estimated by simulation of the two overlapping components.

As was the case for **6**, the proton spectrum, e.g., of the aldehyde signal, indicated small amounts of various double-bond stereoisomers of ***d*-6** with a distribution similar to that of **6**, resulting in overinteger values of the aliphatic chain integrals (12). In view of these factors, we estimated the ***d*-6** content to be at least 82% of all (*E,Z,Z*)-2,4,7-tridecatrienal occurring in the solution. Integration of the aliphatic tail signals showed that (*E,Z,Z*)-2,4,7-tridecatrienal represented at least 80% of all 2,4,7-tridecatrienal present. GC analysis (data not shown) suggested that the stereoisomer (*E,E,Z*)-2,4,7-tridecatrienal is probably the second-most abundant 2,4,7-tridecatrienal in the product.

In the ^{13}C NMR spectra of ***d*-6**, the typical isotope shifts with respect to **6** were found, and the minor signals of remaining nondeuterated carbons 4, 5, 7, and 8 were compatible with the corresponding residual proton integrals found in the ^{1}H NMR spectrum.

The main reason for the isotopic impurities is the well-known phenomenon of deuterium scattering in catalytic deuteration (24). An accurate calculation of isotopic labeling is possible through correction of the data for the naturally occurring deuterium isotopes (24,25). This procedure indicated the following labeling pattern for ***d*-6**: $^2H_4$-**6** (84%), $^2H_3$-**6** (15.3%), $^2H_6$-**6** (0.5%), $^2H_2$-**6** (0.1%), and $^2H_5$-**6** (0.1%). As the isotopic purity is already considered in the calibration curve, mixtures of isotopomers do not preclude the application of IDA as long as the labeled molecules are stable with respect to deuterium/hydrogen exchange. This is the case for all labeled standards used in this study (19–21).

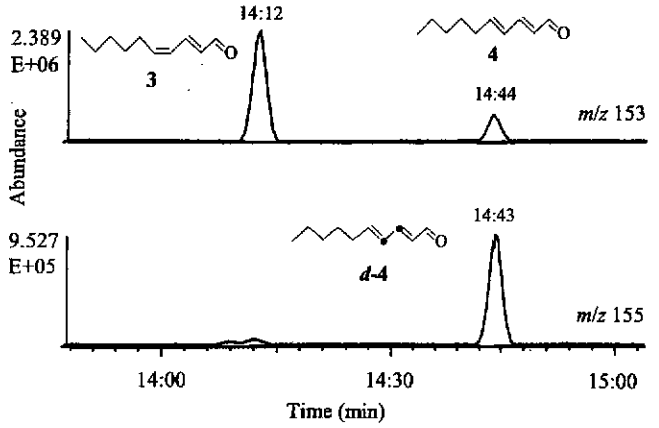


FIG. 2. Quantification of (*E,Z*)-2,4-decadienal (**3**) and (*E,E*)-2,4-decadienal (**4**) by isotope dilution assay using ***d*-4** as internal standard. The $[M + 1]^+$ ions *m/z* 153 of **3** and **4** and *m/z* 155 of ***d*-4** were measured by selective ion monitoring (SIM).

IDA for the quantification of lipid-derived odorants. Characteristic ion pairs of analyte and labeled IS were selectively monitored as shown in Figure 2 for odorants **3** and **4**. As they have almost identical chemical properties, ***d*-4** was used as IS for the quantification of both odorants. The SIM technique was preferably applied to labile compounds, such as odorant **5** (19), particularly if they occurred at low concentrations.

The calibration graphs obtained after plotting ion area ratio vs. amount area ratio showed typical second-order curves (Fig. 3A), particularly when the amount ratios were extended to values higher than 10. This is due to the natural ^{13}C abundance in the analyte, which coincided with the acquired ions of the deuterated IS, i.e., interference of natural isotope enrichment at higher mass or of the isotopic impurity at lower mass (26). To facilitate work within a linear range, we used only the lower part of the calibration curve, which showed excellent linearity (Fig. 3B). Therefore, the amount of IS added to the samples was adjusted to obtain an ion ratio falling in this linear range.

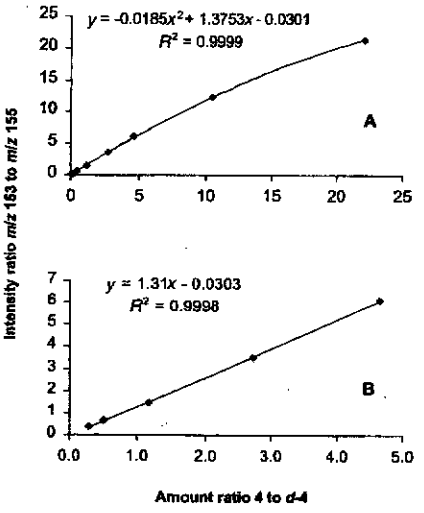


FIG. 3. Calibration curves obtained for the quantification of (*E,E*)-2,4-decadienal (**4**) by isotope dilution assay: (A) second-order curve and (B) linear range used in the quantification experiments.

The accuracy of the measured values compared to the theoretical values was checked according to the procedure recently described (26). Knowing the amount of analyte and labeled IS in the mixture, the theoretical enrichment of ***d*-IS** was calculated for each calibration point (data not shown) and expressed in mol% excess. The measured deuterium enrichment was plotted vs. theoretical deuterium enrichment resulting in linear curves as shown for **5** and ***d*-5** (19).

Quantification of six odorants in autoxidized AA. To obtain reliable quantitative data, concentrations of the potent odorants **1–6** were determined by IDA in the volatile fraction of autoxidized AA. Compound **1** with a green note was the most abundant odorant with about 7.4 g/kg autoxidized AA, which corresponds to 2.2% yield on a molar basis (Table 3). This is in good agreement with literature data reporting hexanal as the major volatile degradation product of autoxidized AA or methyl arachidonate (7,8,10). The concentrations of the remaining odorants varied from about 50 mg/kg AA for **5** (metallic) to nearly 400 mg/kg AA for **3** (fatty) with molar yields of less than 0.1%. The ratio hexanal to 2,4-decadienal was about 16:1, which is higher than those reported in the literature, i.e., 11:1 (7), 5:1 (10), and 1.4:1 (8).

As the concentrations of all of the volatile compounds identified in this study are far above their odor thresholds, they all contribute to the overall aroma of autoxidized AA. The odor activity values (OAV) were calculated as ratio of concentration to threshold. As summarized in Table 3, odorants **3** and **5** showed the highest OAV based on odor thresholds in air. They can be seen as the character impact odorants of autoxidized AA imparting metallic and fatty notes, which were found to be the main odor characters of the authentic sample (11). Data obtained on the basis of odor thresholds in oil indicated that in addition to **3** and **5**, odorants **1** and **2** significantly contribute with green and mushroom notes, respectively.

The significant contribution of **5** is due to its extremely low odor thresholds of 1.5 pg/L air (30) and 1.3 µg/L oil (14). Therefore, despite the lowest amounts found among the six impact odorants (Table 3), its sensory contribution is pronounced. Because of the low threshold value of **3**, particularly in oil (4 µg/L), and relatively high concentration, the sensory contribution of **3** to the overall aroma of autoxidized AA is of similar importance.

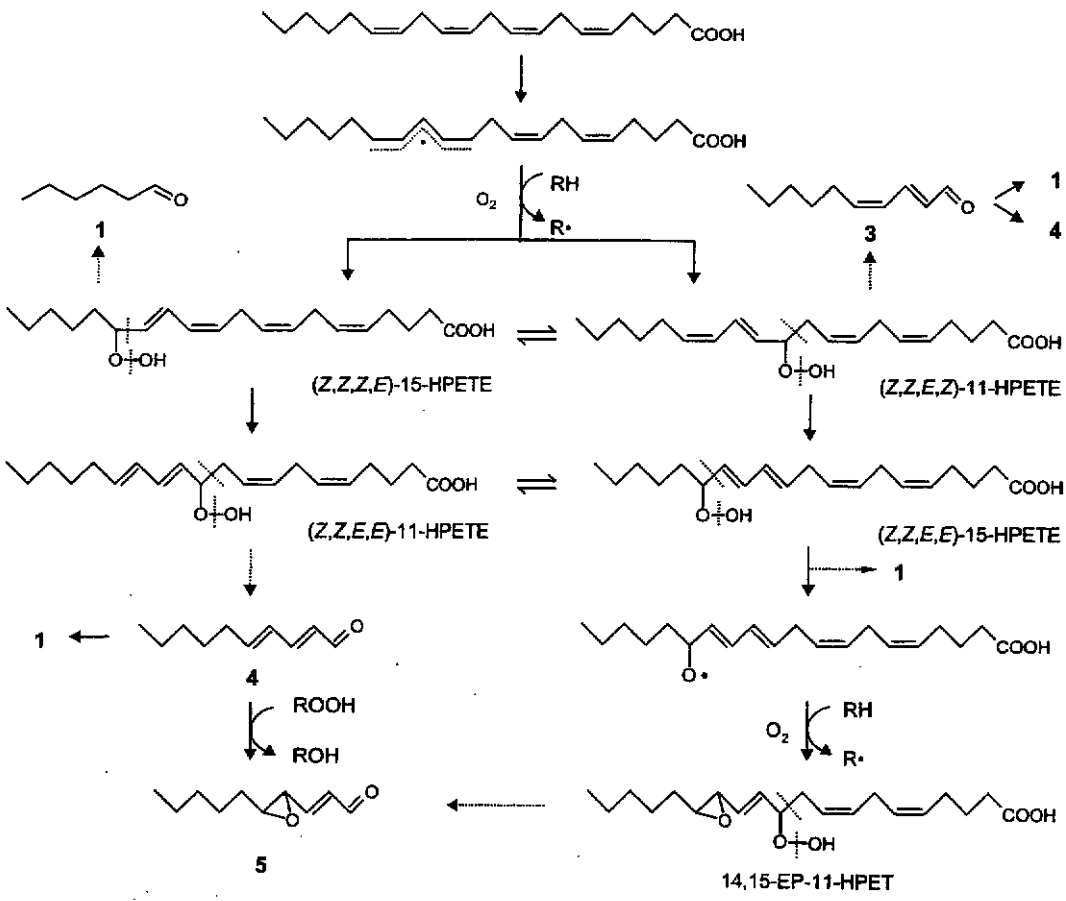
Formation pathways. Since AA belongs to the ω-6 fatty acid family, the formation of odorants with 10 carbon atoms and less can be explained in analogy to linoleic acid (6). Hexanal (**1**) is formed by β-cleavage of 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) as shown in Scheme 2. It was found to be the major odor-active volatile degradation product in autoxidized AA with 2.2% molar yield (Table 3). Its abundance can be explained on the one hand by the relatively high yields of 15-HPETE (~35%) as reported by Yamagata *et al.* (32). On the other hand, odorant **1** is also known to be a secondary autoxidation product of 2,4-decadienal, compounds **3** and **4**, in Scheme 2 (33,34).

TABLE 3
Odor Activity Values (OAV) of Potent Odorants Found in Autoxidized Arachidonic Acid (AA) Obtained by Calculating the Ratio of Concentration and Odor Thresholds in Air and Oil

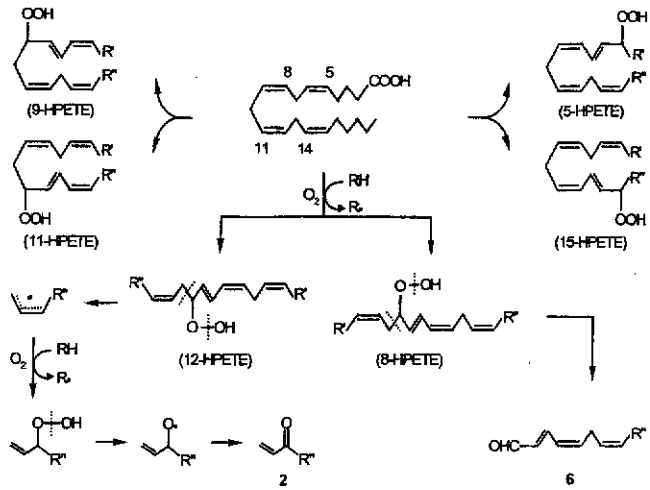
Odorant	Aroma quality	Concentration (mg/kg AA)	Yield (mol%)	Odor threshold (ng/L air)[a]	OAV (air; × 10^6)	Odor threshold (μg/L oil)[b]	OAV (oil)
1	Green	7370 ± 520	2.2	30	245	300	24,570
2	Mushroom-like	70 ± 10	0.02	0.07	1000	10	7,000
3	Fatty	373 ± 7	0.07	0.01[c]	37300	4	93,250
4	Fatty	96 ± 5	0.02	0.1	960	180	530
5	Metallic	47 ± 6	0.008	0.0015	31330	1.3	36,150
6	Egg white-like	131 ± 7	0.02	0.07[c]	1870	180[d]	730

[a]Odor thresholds in air were taken from the literature: **1** (27), **2** (28), **4** (29), **5** (30).
[b]Odor thresholds in oil were taken from the literature: **1** (14), **2** (14), **3** (31), **4** (14), **5** (14).
[c]Odor threshold was determined in this work (30).
[d]Odor threshold of **6** in oil was estimated to be similar to that of odorant **4**.

β-Cleavage of 11-(*Z,Z,E,Z*)-5,8,12,14-HPETE results in compound **3** with the *E,Z*-configuration of the double bonds (Scheme 2). In analogy, odorant **4** can be formed from 11-(*Z,Z,E,E*)-5,8,12,14-HPETE. The lower molar yields of **3** (0.07%) and **4** (0.02%) compared to **1** (2.2%) may be due to the lower amounts of the direct precursor 11-HPETE (~10%) formed by autoxidation of AA (32). The higher yields of **3** compared to **4** might be explained by the fact that **3** is directly formed from the primary precursor 11-(*Z,Z,E,Z*)-5,8,12,14-HPETE. In contrast, the precursor of **4**, 11-(*Z,Z,E,E*)-5,8,12,14-HPETE, must first be generated through isomerization of 11-(*Z,Z,E,Z*)-5,8,12,14-HPETE to 15-(*Z,Z,Z,E*)-5,8,11,13-HPETE. Following the mechanistic study of Porter and Wujek (35), the (*E,E*)-diene isomerization occurs after β-scission of O_2 from the 15-peroxyl radical (not shown in Scheme 2) leading to a pentadiene radical with 13,14 (*E*) and 11,12 (*Z*) conformation. Oxygenation of C-11 gives the desired 11-(*Z,Z,E,E*)-5,8,12,14-HPETE. Furthermore, aldehyde



SCHEME 2



SCHEME 3

4 can also originate from **3**. However, **3** is stable at room temperature and in lipophilic media; isomerization to **4** occurs preferably upon heating or in aqueous solutions.

For the formation of **5**, we suggest 14,15-epoxy-11-hydroperoxy-5,8,12-eicosatrienoic acid (14,15-EP-11-HPET) (Scheme 2) as a key intermediate, in analogy to the formation of **5** from autoxidized linoleic acid (36). Alternatively, **5** can also be formed as a secondary oxidation product of 2,4-decadienal as recently reported by Gassenmeier and Schieberle (36). These authors demonstrated that **5** was preferentially formed from 9-hydroperoxy-10,12-octadecadienoic acid (9-HPOD) *via* 2,4-decadienal as the intermediate. Alternatively, **5** may also be formed by oxidation of **4** with peracids (not shown in Scheme 2).

Formation of **6** can be explained by β-cleavage of the corresponding 8-hydroperoxy-5,9,11,14-eicosatetraenoic acid (8-HPETE) as shown in Scheme 3. This is in analogy to the formation of (*E,Z,Z*)-2,4,7-decatrienal by autoxidation of linolenic acid (8,37). Similar to the formation of **2** in autoxidized linoleic acid from 10-hydroperoxy-8,12-octadecadienoic acid (10-HPOD), the mushroom-smelling odorant can also be generated from AA *via* the alkoxyl radical formed from 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) as shown in Scheme 3, where R′ represents CH_2-CH_2-CH_2-COOH and R″ stands for CH_2-CH_2-CH_2-CH_2-CH_3 (8,37). However, the alkoxyl radical can also lead to 1-octen-3-ol (38) that was detected in autoxidized AA as well (11,12).

Conclusions. Quantitative characterization of the aroma composition of autoxidized AA by isotope dilution assay and calculation of the OAV suggest (*E,Z*)-2,4-decadienal and *trans*-4,5-epoxy-(*E*)-decenal as the character impact odorants, smelling fatty and metallic, respectively. These aroma notes are representative of the overall aroma of autoxidized AA. Hexanal seems to play an important role, particularly in oily media, due to its low odor threshold in oil. The quantitative data obtained for the six odorants are in good agreement with the possible formation mechanisms and the relative amounts of the corresponding hydroperoxides reported in the literature (32).

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