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Characterization of (*E*,*E*,*Z*)-2,4,6-Nonatrienal as a Character Impact Aroma Compound of Oat Flakes

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To identify the compounds evoking the characteristic cereal-like, sweet aroma of oat flakes, an aroma extract dilution analysis (AEDA) was applied to a distillate prepared by solvent extraction/vacuum distillation from commercial oat flakes. Among the nine aroma-active compounds detected by gas chromatography−olfactometry and AEDA in the flavor dilution (FD) factor range of 4−1024, eight odorants, for example, (*E*)-β-damascenone, (*Z*)-3-hexenal, and butanoic acid, showed only low FD factors. However, one odorant eliciting the typical cereal, sweet aroma of the flakes was detected with the highest FD factor of 1024. By mass spectrometry and nuclear magnetic resonance measurements followed by a synthesis, (*E*,*E*,*Z*)-2,4,6-nonatrienal, exhibiting an intense oat flake-like odor at the extremely low odor threshold of 0.0002 ng/L in air, was identified as the key odorant of the flakes. By means of a newly developed stable isotope dilution analysis using synthesized, carbon-13-labeled nonatrienal as the internal standard, a concentration of 13 μg of (*E*,*E*,*Z*)-2,4,6-nonatrienal per kilogram of the flakes was measured. Model studies suggested linolenic acid as the precursor of nonatrienal in oats.

KEYWORDS: (*E*,*E*,*Z*)-2,4,6-Nonatrienal; (*E*,*Z*,*E*)-2,4,6-nonatrienal; precursor; linolenic acid; oats; stable isotope dilution analysis; aroma extract dilution analysis

INTRODUCTION

Cereal grains constitute the major part of human diets throughout the world. Although the world production of oats is minor as compared to that of wheat or rice, there is growing interest in oats because of the higher content of, for example, unsaturated lipids or vitamin B_1 (*1*). However, because of the comparatively higher activity of lipases and lipoxygenases in oats, the kernels have to be thermally treated during manufacturing to prevent an enzymatic lipid peroxidation.

Heydanek and McGorrin (*2*) were among the first to characterize volatiles inherent in dried oat groats prior to processing. On the basis of vacuum distillation of 8 kg of hydrated oat groats, the authors reported the identification of about 100 volatile compounds. Further studies were focused on the volatile composition of oils isolated from crude and roasted oats (*3*, *4*), oatmeal porridge (*5*), extrusion cooked oats (*6*−*8*), or rolled oats (*9*). Besides inactivating the enzymes, the heat treatment is also considered to be necessary to develop the typical nutty aroma of oat products (*5*), and Heydanek and McGorrin (*10*) found an increase in typical Maillard-derived compounds, such as pyrazines and furanones, after the heat treatment of oats.

It is widely accepted in modern aroma analysis that only those volatiles present above their odor thresholds contribute to a given food aroma (*11*−*13*). Such aroma-active components can be separated from the bulk of odorless volatiles by screening methods, such as the aroma extract dilution analysis (AEDA) (*13*). However, applications of such dilution to odor threshold methods on oat volatiles are scarcely available. In a study aimed at identifying aroma compounds causing an off-odor in extruded oat flour after storage, Guth and Grosch (*14*) previously reported on vanillin, *trans*-4,5-epoxy-(*E*)-2-decenal, and (*E*,*E*)-2,4-deca-dienal followed by (*E*,*E*)-2,4-nonadienal, *trans*-2,3-epoxyoctanal, octanal, and hexanal as the most odor-active compounds among the 25 aroma compounds detected in the freshly extruded product. During storage of the dried extrudate, in particular, hexanal and hexanoic acid were much increased and, therefore, the authors suggested these compounds to mainly cause the green, rancid off-note that had developed in the stored product.

Because, to our knowledge, no data are available on the aroma compounds causing the characteristic, sweet, nutty, cereal-like aroma of fresh oat flakes, the purpose of this study was to identify these on the basis of an application of the AEDA.

MATERIALS AND METHODS

Material. Three different batches of oat flakes (Kölln, Elmshorn, Germany) were purchased from a local supermarket.

Chemicals. Samples of the following pure reference chemicals were obtained from the commercial sources given in parentheses: acetic acid, (*E*,*E*)-2,4-heptadienal, 3-methylbutanoic acid, methyl octanoate, vanillin,

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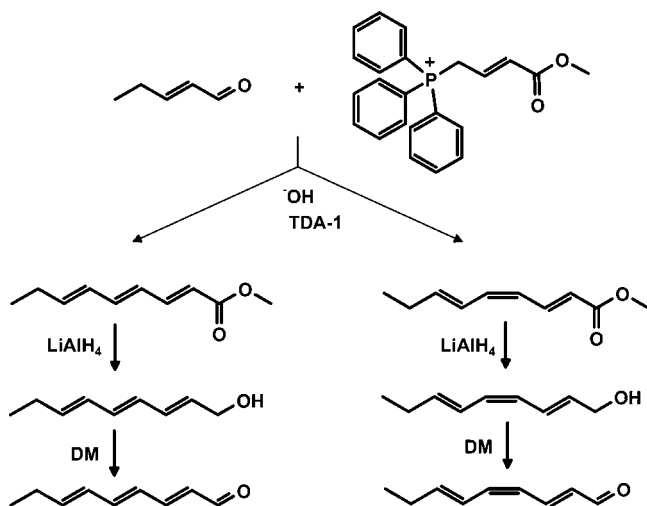
Figure 1. Synthetic route used in the synthesis of (*E,E,E*)- and (*E,Z,E*)-2,4,6-nonatrienal (DM, Dess–Martin periodinane).

(*E*)-2-pentenal, (*Z*)-2-penten-1-ol, (*E,E*)-2,4-heptadienal, [$^{13}C_2$]acetaldehyde, and (*E,Z*)-2,6-nonadienal (Aldrich, Steinheim, Germany); butanoic acid and linolenic acid (Fluka, Neu-Ulm, Germany); 1-octen-3-one, tris[2-(2-methoxyethoxy)ethyl]amine, methyl-4-(triphenylphosphonium)crotonate bromide, and Dess—Martin periodinane (Lancaster, Mühlheim, Germany). (*E*)-β-Damascenone was a gift from Symrise (Holzminden, Germany). All other chemicals were of analytical grade.

(*Z*)-3-Hexenal was synthesized as previously described (*15*).

Syntheses. *(E,E,E)-2,4,6-Nonatrienal.* The synthesis was performed via an aldol condensation of (*E,E*)-2,4-heptadienal and acetaldehyde following closely the method described by Buttery (*16*).

(E,Z,E)-2,4,6-Nonatrienal. The synthetic procedure used for the preparation of this nonatrienal isomer is displayed in **Figure 1**.

In a first step, a mixture of methyl (*E,E,E*)- and (*E,Z,E*)-2,4,6-nonatrienoate was synthesized using a modified Wittig reaction and following a procedure described for cinnamic acid derivatives (*17*). Tris[2-(2-methoxyethoxy)ethyl]amine (TDA-1; 323 mg; 1 mmol), 80 mL of aqueous saturated sodium bicarbonate, and 80 mL of methylene chloride were combined and intensely stirred for several minutes. Then, methyl-4-(triphenylphosphonium)crotonate bromide (1.5 mmol, 662 mg) and (*E*)-2-pentenal (1 mmol, 84 mg) were added, and the mixture was stirred for 12 h at room temperature in the dark. The organic layer was separated, and the aqueous phase was extracted twice with methylene chloride (total volume = 100 mL). The combined organic phases were acidified using hydrochloric acid (75 mL, 10%), and the solution was vigorously stirred for 12 h at room temperature in the dark. The organic layer was separated, and the aqueous phase was extracted twice with methylene chloride (100 mL). The combined organic layers were finally dried over anhydrous sodium sulfate and then concentrated to 2 mL. The solution was placed on the top of a water-cooled glass column (30 × 1.5 cm i.d.) filled with silica, modified according to the method of Esterbauer (*18*), in *n*-pentane. After the column had been flushed with *n*-pentane (50 mL), the target compound was eluted with *n*-pentane/diethyl ether (8 + 2 by vol; 200 mL). The solvent was evaporated at 35 °C to obtain a final volume of 10 mL.

The methyl esters obtained were then reduced into the corresponding alcohols (*E,E,E*)- and (*E,Z,E*)-2,4,6-nonatrien-1-ol, using LiAlH₄. After 1 h of stirring at room temperature in the dark, a few drops of water were added with continuous stirring. When the H₂ production stopped, sulfuric acid (10%, w/w) was added until the precipitate formed was dissolved. The organic layer, containing the alcohols, was separated and dried over anhydrous sodium sulfate.

In the third step (**Figure 1**), the alcohols were oxidized into a mixture of (*E,E,E*)- and (*E,Z,E*)-2,4,6-nonatrienal by means of Dess—Martin periodinane (1 mmol, 440 mg). After the addition of the periodinane, the solution was stirred for 12 h at room temperature in the dark. The solution was concentrated to 200 μL at 35 °C, the suspension was extracted with *n*-pentane (2 mL) and filtered.

(E,E,Z)-2,4,6-Nonatrienal. (*Z*)-2-Penten-1-ol (1.5 mmol, 130 mg) and Dess—Martin periodinane (2 mmol, 880 mg) were dissolved in methylene chloride (10 mL), and the solution was stirred for 12 h at room temperature. Then, the solvent was evaporated at 35 °C, the residue was dissolved in pentane (2 mL), and the remaining residue was filtered off. The solution containing the (*Z*)-2-pentenal was used without further purification.

The further reaction steps were performed as described above for (*E,E,E*)- and (*E,Z,E*)-2,4,6-nonatrienal (cf. **Figure 1**).

Preparative Isolation of the 2,4,6-Nonatrienal Isomers. The mixture of isomers obtained in both synthetic routes was first purified by means of column chromatography. For this purpose, the solution (1 mL) containing the nonatrienals was placed on the top of a glass column (30 × 1.5 cm) filled with a slurry of silica (modified according to the method of ref *18*) in *n*-pentane. After the column had been flushed with *n*-pentane (100 mL), the target compounds were eluted with *n*-pentane/diethyl ether (200 mL, 4:1, v/v).

The isomers were separated by means of preparative thin-layer chromatography on silica gel (60 mesh, 20 × 20 cm, fluorescence indicator F254, focusing zone of 20 × 2.5 cm, thickness = 0.5 mm; Merck, Darmstadt, Germany) using a mixture of *n*-pentane and diethyl ether (9:1, v/v) as the mobile phase. The isomers were detected by UV adsorption and collected separately by scraping off the respective areas from the plate, followed by extraction with diethyl ether.

Quantitation of each isomer, collected in a volumetric flask of defined volume, was performed by GC-FID using methyl octanoate as the internal standard. The results were corrected by an FID response factor obtained for a mixture of methyl octanoate and (*E,Z*)-2,6-nonadienal.

[$^{13}C_2$]-(E,E,E)-2,4,6-Nonatrienal. Synthesis of [$^{13}C_2$]-(*E,E,E*)-2,4,6-nonatrienal ([$^{13}C_2$]-NT) was performed following closely the procedure described for the unlabeled compound (*16*).

(*E,E*)-2,4-Heptadienal (0.6 mg, 6 mmol) was cooled at −5 °C in an ice bath. After the addition of [$^{13}C_2$]acetaldehyde (0.55 mg, 12 mmol) and potassium hydroxide (0.5 mL; 50%, v/v), the mixture was stirred at −5 °C for 15 min and then for 1 h at room temperature. Diethyl ether (100 mL) was added, and the mixture was washed with hydrochloric acid (50 mL, 3 mol/L), followed by aqueous saturated sodium bicarbonate solution (50 mL). Finally, the organic layer was dried over anhydrous sodium sulfate. The concentration was determined using methyl octanoate as the internal standard using the response factor determined for the unlabeled compound.

Isolation of Volatiles from Oat Flakes. Oat flakes (50 g) were powdered in liquid nitrogen using a Waring Blendor. The powder was repeatedly extracted with diethyl ether (total extraction time = 2 h; total volume = 300 mL). The combined extracts were filtered, dried over anhydrous sodium sulfate, and concentrated to 100 mL by distilling off the solvent at 40 °C using a Vigreux column (50 × 1 cm). To remove the nonvolatile material, the extract was distilled at 40 °C using the solvent-assisted flavor evaporation (SAFE) technique (*19*). Finally, the distillate was concentrated to 0.5 mL at 40 °C by means of a Vigreux column (50 × 1 cm), followed by microdistillation.

High-Resolution Gas Chromatography−Olfactometry (HRGC-O); AEDA. HRGC was performed by means of a gas chromatograph type 5160 (Fisons Instruments, Mainz, Germany) using the following fused silica capillary columns: DB-5 (30 m × 0.32 mm, film thickness = 0.25 μm) (J&W Scientific, Folsom, CA), DB-FFAP (30 m × 0.32 mm, film thickness = 0.25 μm) (Phenomenex, Folsom, CA). The samples were injected at 40 °C using the cold on-column technique. The oven was held at 40 °C for 2 min, and the temperature was then raised to 150 °C at 6 °C/min and then to 230 °C at 20 °C/min. For HRGC-O, the effluent was split evenly between a flame ionization detector (FID) and a sniffing port. Retention indices (RI) were calculated from the retention times of *n*-alkanes by linear interpolation. For AEDA (*13*, *14*), extracts were diluted stepwise with diethyl ether (1:1, v/v) and each dilution was analyzed by HRGC-O (injection volume = 0.5 μL).

Mass Spectrometry (MS). For compound identification, the effluent of the GC column was introduced by means of the open split mode into the mass spectrometer, and mass spectra were generated using the MAT 95 S (Finnigan, Bremen, Germany) at 70 eV in the electronic

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ionization mode (MS/EI) and at 115 eV in the chemical ionization mode (MS/CI; reagent gas was isobutane).

Quantitation of 2,4,6-Nonatrienal Isomers in Oat Flakes. Quantitation was performed by means of a newly developed stable isotope dilution assay. For this purpose, $[^{13}C_2]$-(E,E,E)-2,4,6-nonatrienal ($[^{13}C_2]$-NT) was synthesized and, first an MS response factor of 0.99 was determined from five different model solutions containing known, but differing, amounts of the unlabeled and labeled nonatrienal. The ions m/z 137 (for 2,4,6-nonatrienal) and m/z 139 (for $[^{13}C_2]$-2,4,6-nonatrienal) were used.

For quantitation, $[^{13}C_2]$NT (5 μg) was added to powdered oat flakes (200 g), and the volatiles and the labeled standard were isolated by extraction and SAFE distillation as described above. For two-dimensional GC-MS a Mega 2 series gas chromatograph (Fisons Instruments, Mainz-Kastel, Germany) was coupled to a Mega series 5160 gas chromatograph (Carlo Erba, Hofheim, Germany) via the MCCS system (Fisons Instruments). MS analysis was performed by means of an ITD-800 mass spectrometer (Finnigan MAT; Bremen, Germany), which was operated in the chemical ionization mode using methanol as reactant gas. The separation of the distillate was accomplished by using a DB-5 (first oven) in combination with an FFAP column (second oven), following the GC oven program outlined above. The cut time intervals were determined by injection of the reference compound prior to the analysis of the samples.

Model Experiment on 2,4,6-Nonatrienal Formation from Linolenic Acid. Linolenic acid (100 mg) was dissolved in 10 mL of water using 5 mL of an aqueous solution of the emulsifier Tween 80 (0.001%) and sodium hydroxide (1 mol/L; 0.1 mL). The solution was poured into phosphate buffer (185 mL, 0.1 mol/L, pH 5.5), flushed with pure oxygen for 5 min, and stirred for 14 h at 40 °C in a closed vessel. After incubation, the volatiles were isolated by extracting the solution twice with methylene chloride (50 mL) followed by SAFE distillation of the dried extract.

Additionally, a blank sample using the same mixture, but without incubation, was analyzed. The quantitation of nonatrienal was performed as described above for oat flakes.

Proton Magnetic Resonance Spectrometry. NMR spectra were recorded in CDCl₃ solution by means of a Bruker AMX 400-III (Bruker, Rheinstetten, Germany; 400 MHz) spectrometer using tetramethylsilane as the internal standard.

RESULTS AND DISCUSSION

Odorants in Oat Flakes. First, an overall sensory evaluation of a distillate prepared by extraction of oat flakes with diethyl ether followed by SAFE distillation was performed by 10 experienced panelists. On the basis of results obtained by smelling an aliquot of a distillate on a strip of filter paper, they characterized the aroma of the extract to be very close to the characteristic cereal, nutty aroma of the flakes.

To characterize the volatile constituents responsible for the overall odor impression, the distillate was separated by high-resolution gas chromatography and the eluate was sniffed by four assessors using an odor port (GC-O). The "free fatty acid phase" (FFAP) was used to enable a separation of acidic and neutral/basic volatiles in one GC run. GC-O revealed a total of only nine odor-active areas; surprisingly, two of these clearly revealed on odor quality similar to the overall aroma of the oat flakes. By application of the AEDA, one of the two aroma compounds was found to reveal by far the highest FD factor among all odor-active areas detected.

A comparison of retention indices and odor qualities of the odor-active areas detected with those of an in-house database prepared by using reference odorants (*20*) suggested chemical structures for seven of the nine odor-active areas. Finally, a comparison of mass spectrometric data obtained for the oat constituent and the respective reference compound confirmed (E)-β-damascenone, (Z)-3-hexenal, acetic acid, butanoic acid, 1-octen-3-one, vanillin, and 3-methylbutanoic acid

Table 1. Important Aroma Compounds (FD ≥ 4) Identified in an Extract of Oatmeal Flakes

no.	odorant[a]	odor quality	RI on FFAP[c]	FD[d]
1	(Z)-3-hexenal	green, grassy	1148	8
2	1-octen-3-one	mushroom-like	1298	4
3	acetic acid	sour	1430	8
4	butanoic acid	sweaty, rancid	1625	8
5	3-methylbutanoic acid	sweaty	1663	4
6	(E)-β-damascenone	cooked apple	1815	16
7	unknown	oatmeal-like, sweet	1869	8
8	unknown	oatmeal-like, sweet	1877	1024
9	vanillin	vanilla-like	2605	4

[a] The compound was identified by comparing the mass spectra (MS/EI and MS/CI) and retention indices on three columns of different polarities as well as the odor quality and odor threshold with the respective data obtained from the reference compound. [b] Odor quality of the food constituent and the respective reference compound as perceived at the sniffing port. [c] Retention index. [d] Flavor dilution factor.
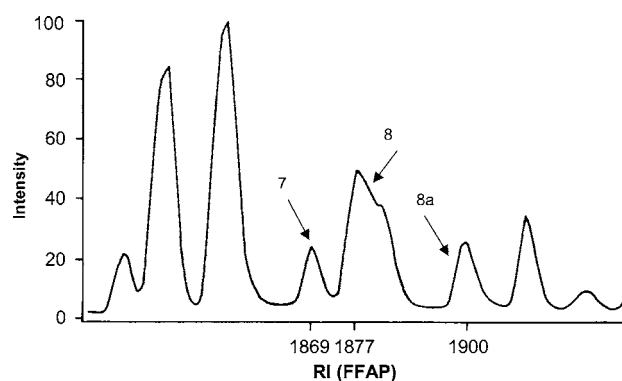
Figure 2. HRGC separation of column fraction II isolated from a distillate of oat flakes. Arrows indicate the retention indices of compounds **7** and **8** as well as of **8a** showing identical mass spectra.

as odor-active constituents in oat flakes (**Table 1**). However, for the two compounds eliciting oatmeal-like aromas (**7** and **8**), no reference compound was available and, also, no clear mass spectrum could be obtained in the extract prepared from 50 g of oat flakes, suggesting both compounds to be very odor-active.

Thus, to obtain an unequivocal mass spectrum, the volatiles from 1 kg of oat flakes were isolated, both odorants were enriched by column chromatography on silica gel using an *n*-pentane/diethyl ether gradient, and the odor-active region in the gas chromatogram of the respective fraction was accumulated in a cooled trap by two-dimensional HRGC and by repeatedly injecting the extract. In **Figure 2**, the gas chromatogram containing the oat-like smelling compound is shown. By GC-O, compounds **7** and **8** were clearly located at retention indices of 1869 and 1877. In both areas, the same mass spectrum (MS/EI) was obtained (**Figure 3A**). In addition, a nearly identical mass spectrum (**Figure 3B**) was obtained for a third peak (compound **8a**) eluting at RI 1900 (**Figure 2**), which, however, did not have an aroma. In all three compounds, the ions m/z 136 were confirmed as the molecular mass by MS/CI. High-resolution mass spectrometry revealed the elemental composition $C_9H_{12}O$, which indicated the presence of four double-bond equivalents.

A thorough review of the literature identified a study by Buttery (*16*) on volatiles of dry beans, in which the author reported on (E,E,E)-2,4,6-nonatrienal as the first identification of this volatile compound as a food constituent. The mass spectrum published by Buttery (*16*) was in good agreement with
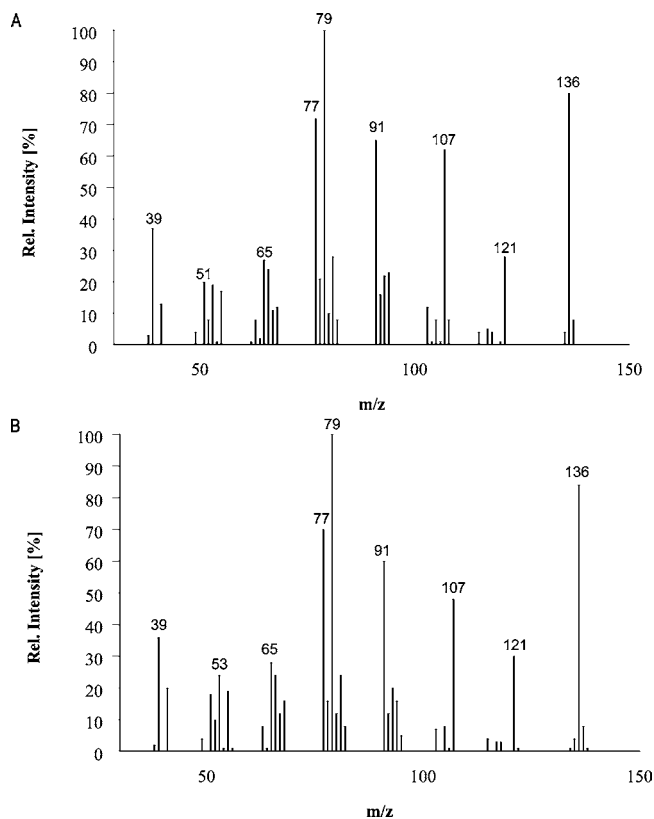
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Figure 3. Mass spectra (MS/EI) of (**A**) compounds **7** and **8** and (**B**) compound **8a**.

Figure 4. HRGC separation of (**A**) two isomers of 2,4,6-nonatrienal prepared by starting from (*E*)-2-pentenal (cf. **Figure 1**) and (**B**) nonatrienal isomers obtained in a synthesis starting from (*Z*)-2-pentenal instead of (*E*)-2-pentenal (cf. **Figure 1**).

the spectrum shown in **Figure 3B**. However, no data on the aroma quality of the nonatrienal nor on its GC retention index were published (*16*). Further to this study, another six publications had previously reported on the presence of 2,4,6-nonatrienal in different foods, such as endive (*21*), lamb's lettuce (*22*), curuba fruit (*23*), orange juice (*24*), anchovies (*25*), and spinach (*26*).

However, also in these studies no data on the correct structure of the respective isomers were published, for example, by performing a synthesis or NMR measurements, nor have any data on the respective odor attributes been published yet. Therefore, first, the (*E,E,E*)-2,4,6-nonatrienal was synthesized following closely the procedure described by Buttery (*16*). The compound obtained showed the same mass spectrum displayed in **Figure 3B**, and a retention index of 1900 was determined on the DB-FFAP column.

Because compounds **7** and **8** were eluted at retention indices of 1869 and 1877, respectively, on this column (**Figure 2**), it was shown that (*E,E,E*)-2,4,6-nonatrienal was present in oats, but it could be excluded that this isomer contributed to the aroma of the flakes. On the other hand, the data suggested that **7** and **8** are possibly geometrical isomers of the *E,E,E* isomer, but having at least one *Z* double bond in the molecule, because of the lower retention index.

In total, eight isomers of 2,4,6-nonatrienal may occur. Because, except for the *E,E,E* isomer, no data are available in the literature on the other isomers, synthetic experiments were performed. Because an *E* configuration in the 2-position might be most probable due to the higher thermal stability of, for example, (*E*)-2-alkenals, first, the synthesis of (*E,Z,E*)-2,4,6-nonatrienal was attempted on the basis of the reaction sequence shown in **Figure 1**.

As shown, a Wittig-type reaction of (*E*)-2-pentenal and methyl-4-(triphenylphosphonium) crotonate should result in the
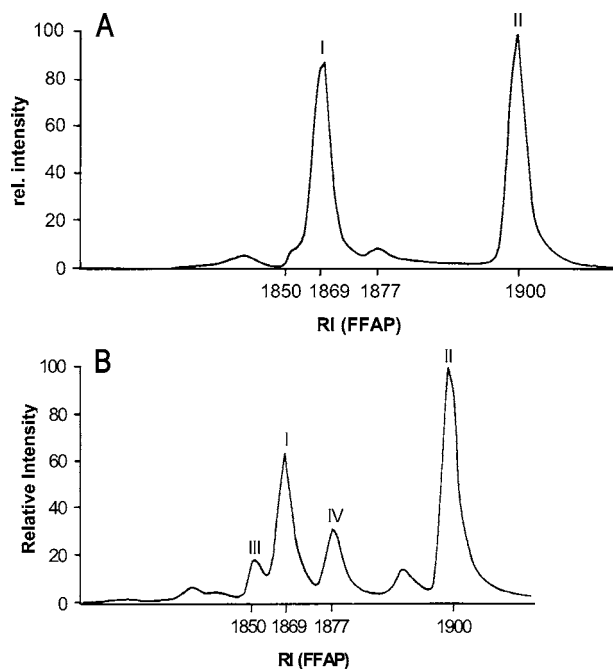
formation of a mixture of methyl (*E,E,E*)- and (*E,Z,E*)-2,4,6-nonatrienoate, which, after reduction into the corresponding alcohols followed by oxidation, should yield two isomers of nonatrienal, namely, the *E,E,E* and *E,Z,E* isomers.

Synthesis following this sequence resulted in two peaks in a 1:1 ratio (**Figure 4A**). Whereas peak II agreed with (*E,E,E*)-2,4,6-nonatrienal in its mass spectrum and retention index, peak I showed the same retention index and mass spectrum as odorant **7** in oat flakes (**Table 1**), thus suggesting its structure to be (*E,Z,E*)-2,4,6-nonatrienal.

For NMR measurements, both isomers were isolated by thin-layer chromatography and were directly taken up in the solvent used for NMR measurements. In a first experiment, an H,H-homonuclear correlation spectroscopy was done with peak II to establish the correct correlation of H atoms and the chemical shifts. Then, ^{1}H NMR spectra were measured, in particular, to determine the coupling constants in the area between C-2 and C-6.

In **Table 2**, the ^{1}H NMR data obtained for peaks I and II are contrasted. The ^{1}H NMR data of peak II, the *E,E,E* isomer (**Table 2**), showed coupling constants of 15.2 Hz (J_{2-3}) and 14.8 Hz (J_{4-5}), corroborating the trans configuration at the respective double bonds at C-2 and C-4. In peak II, coupling constants of 15.1 Hz (J_{2-3}) and 11.1 Hz (J_{4-5}) were measured, allowing the conclusion that the double bond at C-2 is trans-configured and that at C-4 cis-configured in this isomer. On the basis of these data, peak I was assigned as (*E,Z,E*)-2,4,6-nonatrienal. Because all data of peak I were in agreement with those of compound **7**, this was identified as (*E,Z,E*)-2,4,6-nonatrienal. The most odor-active isomer, **8** (**Table 1** and **Figure 2**), however, was still unknown.

Following the idea that **8** might be the *E,E,Z* isomer, (*E*)-2-pentenal was substituted by (*Z*)-2-pentenal in the reaction scheme shown in **Figure 1**. The synthetic route should then result in a mixture of (*E,E,Z*)- and (*E,Z,Z*)-2,4,6-nonatrienal.

However, separation of the isomeric mixture of 2,4,6-nonatrienals obtained showed four peaks with the (*E,Z,E*)- and

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Table 2. ^{1}H NMR Data of Synthesized Peaks I, II, and IV (cf. **Figure 4**)

(E,E,E)-2,4,6-nonatrienal (peak II)   (E,Z,E)-2,4,6-nonatrienal (peak I)

(E,E,Z)-2,4,6-nonatrienal (Peak IV)
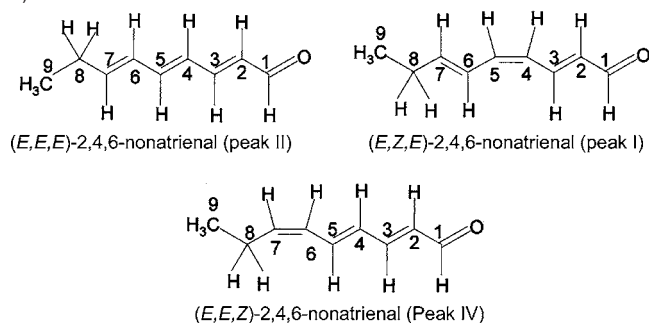
			chemical shift (coupling constants)		
H at C[a]	S[b]	N (H)[c]	peak II	peak I	peak IV
1 (2)	d	1	9.58 (8.0)	9.65 (7.9)	9.55 (7.7)
2, 6, 7	m	3	6.06–6.26	6.05–6.25	6.03–6.23
3 (2/4)	dd	1	7.13 (15.2/11.2)	**7.60** (15.1/10.9)	7.17 (15.2/11.1)
4 (3/5)	dd	1	6.38 (11.1/14.8)	6.43 (10.9/11.1)	6.43 (11.2/14.8)
5 (4/6)	dd	1	6.68 (14.8/10.5)	6.66 (10.2/14.1)	**6.95** (14.8/11.5)
8 (7/9)	quint	2	2.22 (7.1/7.4)	2.28 (7.2/7.5)	2.30 (7.6/7.6)
9 (8)	t	3	1.08 (7.4)	1.15 (7.5)	1.05 (7.4)

[a] Hydrogen at the numbered carbon atom of 2,4,6-nonatrienal; coupling constants (hertz) are given in parentheses. [b] Multiplicity (d = doublet, m = multiplet, dd = double doublet, quint = quintet, t = triplet). [c] Number of hydrogen atoms at the respective carbon atom.

(E,E,E)-2,4,6-nonatrienal as the main constituents (**Figure 4B**). In addition, two further isomers, characterized by the same mass spectrum, were eluted at RI 1851 (peak III) and RI 1877 (peak IV), the latter being identical in its retention index with the most odor-active compound, **8**, in oat flakes (**Table 1**) and, also, showing the most intense odor among the four isomers during GC-O analysis of the synthetic mixture. Because Daubresse et al. (*17*) had already reported that the basic conditions used in the Wittig reaction converted a major part of the *Z* isomers into the more stable *E* isomers, this result was to be expected.

By application of thin-layer chromatography, the (E,Z,E)-2,4,6-nonatrienal (peak I in **Figure 4B**) as well as peak III could be separated from peak IV. However, it was impossible to separate the (E,E,E)-2,4,6-nonatrienal (peak II) from peak IV. In addition, also attempts to separate the isomers of the methyl esters or the alcohols prior to the oxidation step (cf. **Figure 1**) were unsuccessful (data not shown).

For this reason, the ^{1}H NMR measurements were performed on a mixture of peak IV and (E,E,E)-2,4,6-nonatrienal. The signals obtained after subtracting the signals for the *E,E,E* isomer are summarized in **Table 2**. The data confirmed a trans configuration at C-2 and C-4, because coupling constants of 15.2 Hz (J_{3-2}) and 14.8 Hz (J_{4-5}) were measured for peak IV (**Table 2**). Unfortunately, as for all isomers, it was not possible to determine the coupling constant J_{6-7}, because a multiplet was always obtained in this area. However, because the chemical shift of the H atom at C-5 differed significantly from the chemical shift of the H atom at C-5 in the *E,E,E* isomer (**Table 2**), the double bond at C-6 must, consequently, have a cis configuration in peak IV. The same upfield shift was found for the proton at carbon 3, in the (E,Z,E)-2,4,6-nonatrienal.

Thus, compound **8** in oat flakes, agreeing in its retention index with peak IV, was identified as (E,E,Z)-2,4,6-nonatrienal. The elution order of the three isomers (**Figure 2**) on an unpolar stationary GC phase, such as DB-5, clearly followed the well-known rule for α,γ-unsaturated aldehydes with the *E,E* isomer eluting later than the *E,Z* isomer. Although the synthetic pathway

Table 3. Retention Indices, Odor Thresholds, and Odor Qualities of 2,4,6-Nonatrienal Isomers

	retention index on			odor threshold[b]	
isomer[a]	FFAP	DB-5	OV-1701	(ng/L in air)	odor quality
E,Z,E	1869	1271	1433	0.1	oatmeal-like, sweet
E,E,Z	1877	1276	1440	0.0002	oatmeal-like, sweet
E,E,E	1900	1286	1452	4.4	oatmeal-like, sweet

[a] Isomer of 2,4,6-nonatrienal. [b] Odor thresholds were determined as described in ref *14*.
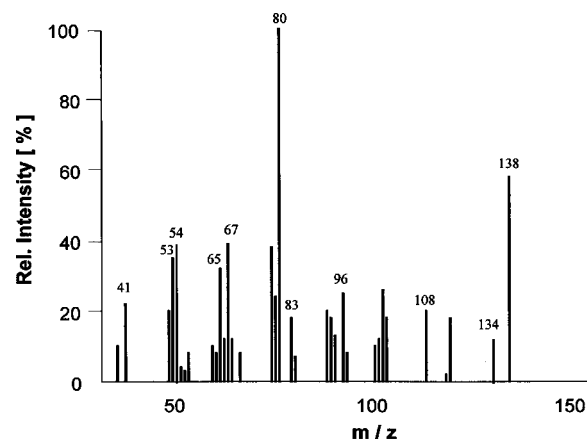
Figure 5. Mass spectrum (MS/EI) of [[13]C$_2$]-(E,E,E)-2,4,6-nonatrienal.

used suggested peak III in **Figure 4** to be the (E,Z,Z)-2,4,6-nonatrienal, the amounts available were not sufficient to unequivocally confirm its structure by ^{1}H NMR.

In **Table 3**, the retention indices and the odor thresholds determined for the three isomers identified in oat flakes are summarized. Although all three isomers elicited the same oat-like, sweet aroma as evaluated by a panel of 10 trained panelists, the odor thresholds determined in air showed significant differences. Although already the odor threshold of the (E,E,E)-2,4,6-nonatrienal was quite low (4.4 ng/L in air), the threshold of the *E,Z,E* isomer was by a factor of 40 lower. The (E,E,Z)-2,4,6-nonatrienal, however, showed the lowest threshold among the three isomers investigated, being by a factor of 20 000 lower as compared to the *E,E,E* isomer. Thus, this compound currently belongs to the most odor-active aroma compounds found in food so far. In higher concentrations, the odor attribute changed to a fatty, deep-fried odor similar to that of (E,E)-2,4-decadienal. Thus, the pleasant oat-flakes-like aroma might be overlooked at the high concentrations present in the air during synthesis.

Quantitation of (E,E,Z)- and (E,E,E)-2,4,6-Nonatrienal in Oat Flakes. Stable isotope dilution assays are an exact tool to quantify trace odorants in foods (*13*). However, the isotopically labeled internal standards are often commercially not available. To develop a quantitation procedure, first, (E,E)-2,4-heptadienal was reacted with [[13]C$_2$]acetaldehyde in an Aldol-type reaction to generate [[13]C$_2$]-(E,E,E)-2,4,6-nonatrienal. The mass spectrum (**Figure 5**) confirms the incorporation of two carbon-13 atoms into the target compound. The isotopomer was purified by chromatography, and a defined mixture of the unlabeled analyte and the labeled standard were analyzed by two-dimensional HRGC-MS as described above. From the mixtures, an MS response factor of 0.99 was calculated.

By applying the newly developed stable isotope dilution assay on three different batches of oat flakes, mean concentrations of 28 μg/kg of the (E,E,E)-2,4,6-nonatrienal and 13 μg/kg of the
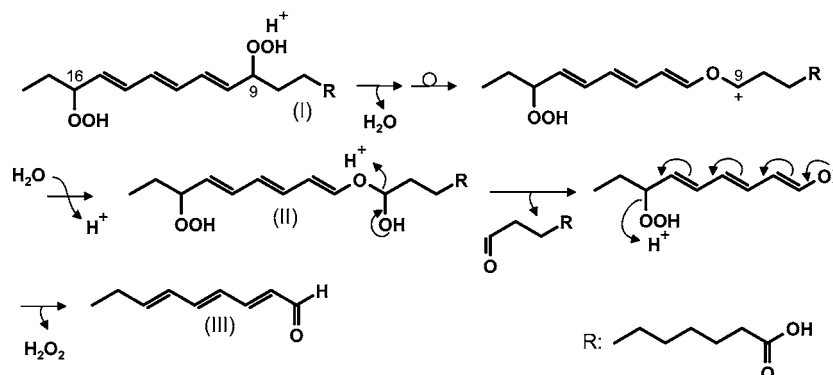
Figure 6. Pathway suggested for nonatrienal formation from a 9,16-dihydroperoxide of linolenic acid.

Table 4. Amounts of (*E*,*E*,*E*)- and (*E*,*E*,*Z*)-2,4,6-Nonatrienal (NT) Formed during Autoxidation of Linolenic Acid (LnA)

	concn (µg/100 mg of LnA)	
expt	(*E*,*E*,*E*)-NT	(*E*,*E*,*Z*)-NT
1	54	30
2	83	52
3	0.3[a]	<0.1[b]
4	0.2[a]	<0.1[b]

[a] Unstored, blank sample. [b] Below detection limit of 0.1 µg/100 mg of LnA.

(*E*,*E*,*Z*)-2,4,6-nonatrienal were determined. The analysis of both compounds in three different batches of oat flakes did not differ more than 15%. Other isomers were generally below the detection limit (0.5 µg/kg, if 200 g of oat flakes is used for extraction).

Because the *E*,*E*,*Z* isomer elicited the lowest odor threshold among all isomers occurring in oats, the quantitative data confirmed this isomer to be the character impact odorant of the flakes.

Studies on the Origin of 2,4,6-Nonatrienal. Buttery (*16*) had already suggested linolenic acid as a precursor of (*E*,*E*,*E*)-2,4,6-nonatrienal, but he had indicated that common pathways leading to such aldehydes, that is, a simple α-cleavage of monohydroperoxides (*27*), would not be able to explain a third double bond. In a study on the oxidation of linolenic acid by soybean lipoxygenase (*28*) or, later, on potato and bean lipoxygenases (*29*), Grosch and Laskawy reported the presence of 2,4,6-nonatrienal using derivatization of the aldehyde with 2,4-dinitrophenylhydrazine.

To investigate whether the nonatrienal isomers might also be formed by an autoxidation of linolenic acid, the unsaturated fatty acid was suspended in aqueous buffer at pH 5.5 and stirred for 14 h at 40 °C. The isomers formed were first analyzed by GC-O and then quantified by means of the stable isotope dilution analysis. The quantitative results obtained (**Table 4**) clearly indicated a significant increase in (*E*,*E*,*E*)- and (*E*,*E*,*Z*)-2,4,6-nonatrienal after 14 h as compared to the very low amounts already present in the linolenic acid before storage. As found for oat flakes, also in the model experiment the *E*,*E*,*E* isomer was higher in its concentration as compared to the *E*,*E*,*Z* isomer. These data proved that nonatrienal can be formed not only by an enzymatic degradation of linolenic acid but also by an autoxidation.

Unsaturated aldehydes, such as 2,4-decadienal, are suggested to be formed from unsaturated fatty acids via a cleavage of alkoxy radicals formed by a homolytic cleavage of monohydroperoxides (*27*). However, up to now, no pathway has been described leading to a conjugated triene system, as in 2,4,6-nonatrienal.

A possible formation pathway may start from dihydroperoxy fatty acids (**Figure 6**). Such peroxides have already been reported by Neff et al. (*30*) and Grechkin et al. (*31*) using either a photosentized peroxidation of methyl linolenate acid or an enzymatic peroxidation of the free acid with potato lipoxygenase. The formation could thus start from the 9,16-dihydroperoxy-10,12,14-octadecatrienoate (**Figure 6**). A proton attack at the 9-hydroperoxy group, followed by water elimination in a Hock-type reaction. may lead to an oxa-cation, which in turn rearranges into a carbonium ion. Addition of water leads to a semiketal, which is split into a triene-hydroperoxide. Enolization and elimination of hydrogen peroxide finally result in 2,4,6-nonatrienal.

Because the homolytic cleavage of hydroperoxides is also discussed for hydroperoxide lyase reactions, it can thus be explained that the nonatrienal can be formed either by an autoxidation leading to dihydroperoxides followed by a heterolytic cleavage of one hydroperoxy group or, alternatively, by enzymatic degradation pathways.

Very recently, we were also able to identify (*E*,*E*,*Z*)-2,4,6-nonatrienal as an important odorant in black tea (Schuh and Schieberle, unpublished results), suggesting that this odorant may occur ubiquitously in foods containing linolenic acid.

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