# Development of a Stable Isotope Dilution Assay for the Quantification of 5-Methyl-(*E*)-2-hepten-4-one: Application to Hazelnut Oils and Hazelnuts

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A stable isotope dilution assay was developed for the quantitation of the hazelnut odorant 5-methyl-(*E*)-2-hepten-4-one by mass chromatography using synthesized $[^2H]_2$-5-methyl-(*E*)-2-hepten-4-one as the internal standard. Application of the method on two batches of commercial hazelnut oils, processed from either roasted or unroasted nuts, revealed 6.4 $\mu$g 5-methyl-(*E*)-2-hepten-4-one per kg of unroasted oil whereas 315.8 $\mu$g per kg was determined in the roasted nut oil. The about 50-fold higher amount of 5-methyl-(*E*)-2-hepten-4-one in roasted hazelnut oil suggested the necessity of a thermal treatment to generate the flavor compound. Pan frying of raw hazelnuts (9 to 15 min) or boiling of the crushed nut material for 1 h in water led to an increase of 5-methyl-(*E*)-2-hepten-4-one by factors of 600 and 800, respectively, thereby corroborating that the major part of the nut flavorant is formed during heat treatment from a yet unknown precursor in hazelnuts.

**Keywords:** *Stable isotope dilution assay; 5-methyl-(E)-2-hepten-4-one; hazelnuts*

## INTRODUCTION

5-Methyl-(*E*)-2-hepten-4-one (MHO), showing an intense, hazelnut-like aroma at the very low odor threshold of 5 ng/kg oil, was reported for the first time in hazelnuts or a food, respectively, by Emberger (1985). The compound, named "Filbertone", had been enriched from 50 kg of roasted hazelnuts (*Corylus avellana* L.) and was characterized by NMR measurements and synthetic experiments. Later, Güntert et al. (1991) showed that the (*E*)-*S*-diastereoisomer elicited the most typical hazelnut odor among the four possible isomers and also had the lowest odor threshold. Recently, by application of the aroma extract dilution analysis on a flavor distillate isolated from a commercially processed hazelnut oil, MHO was established among the key odorants contributing to the overall flavor of the roasted nuts (Matsui et al., 1998). Very recently MHO has been identified also as an important flavor constituent of milk chocolate (Schnermann and Schieberle, 1997; Pfnuer and Schieberle, 1999).

Using a simultaneous distillation/extraction (SDE) technique to isolate the nut volatiles and 3-decanone as the internal standard, Silberzahn (1988) reported MHO concentrations in the range of 500 to 2000 $\mu$g per kg of unroasted hazelnuts of different varieties. After roasting, the amounts were increased by 80% and, therefore, it was suggested that a major part of MHO is biosynthesized in the nuts, whereas a smaller portion is generated from a yet unknown precursor.

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SDE is known to generate additional amounts of certain odorants because of the cooking temperature applied (Schieberle, 1995a). The aim of the following study was, therefore, to develop a more careful quantitation procedure for MHO using a stable isotope dilution assay based on a deuterium labeled internal standard and to apply the method to raw, unroasted and roasted hazelnut samples. As previously summarized (Schieberle, 1995b), the stable isotope dilution analysis is a powerful tool in the quantitation of food odorants which, e.g., are quite unstable, occur in low concentrations, or are, at least in part, formed from precursors.

## EXPERIMENTAL PROCEDURES

**Materials.** Two batches of commercially cold-processed hazelnut oils and one batch of fresh deshelled hazelnuts were purchased from the local trade and stored at 4 °C until use. Commercial oil samples were labeled as cold-pressed oils from either unroasted or roasted nuts. The oil content of the deshelled hazelnuts was 63% (determined by extraction with diethyl ether).

**Chemicals.** The following chemicals were obtained from Merck (Darmstadt, Germany): 2-methyl-3-buten-1-ol, platinum on charcoal, pyridinium chlorochromate, lithium wire, (*E*)-1-brom-1-propen, dichloromethane, anhydrous diethyl ether. *N*-Pentane was purified as previously reported (Schieberle and Grosch, 1983). Unlabeled 5-methyl-(*E*)-2-hepten-4-one was a gift from Dr. M. Güntert (Haarmann & Reimer, Holzminden, Germany).

**Synthesis of Labeled $[^2H]_2$-5-Methyl-(*E*)-2-hepten-4-one ($[^2H]_2$-MHO).** The deuterium labeled MHO was prepared by a four-step reaction sequence starting from 2-methyl-3-butene-1-ol.

*$[^2H]_2$-2-Methylbutanol.* 2-Methyl-3-buten-1-ol (6 mmol) was deuterated in $D_2O$ with deuterium gas at 500 kPa in a laboratory autoclave (type II, Fa. Roth, Karlsruhe, Germany) using platinum on charcoal (15 mg) as the catalyst. After 150 min, the catalyst was filtered off, the reaction mixture was extracted

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with diethyl ether (3 × 50 mL), then dried over $Na_2SO_4$. After removing the solvent by distillation over a Vigreux column, the tanget compound was obtained in a yield of 95%.

*[$^2$H]$_2$-2-Methylbutanal.* [$^2$H]$_2$-2-Methylbutanol, dissolved in dry dichloromethane (20 mL) was added in one portion to a stirred solution of pyridinium chlorochromate (9.4 mmol) in dry dichloromethane (30 mL). After 90 min, dry diethyl ether (40 mL) was added, the supernatant was decanted, and the insoluble residue was washed three times with diethyl ether (total volume 45 mL). The combined suspensions were filtered over silica gel (1 g) in diethyl ether, and the solution was then concentrated to 10 mL.

*[$^2$H]$_2$-5-Methyl-(E)-2-hepten-4-ol.* (*E*)-1-Lithium-1-propen was prepared from lithium wire (70 mmol) and (*E*)-1-brom-1-propen (60 mmol) in anhydrous diethyl ether according to Tao et al. (1974). [$^2$H]$_2$-2-Methylbutanal was added dropwise to the ice-cooled solution, and the mixture stirred for 1 h under an atmosphere of pure argon. Water (20 mL) was added stepwise, the mixture was extracted with diethyl ether (3 × 20 mL) and dried over $Na_2SO_4$. The organic phase was concentrated to 1 mL and separated by flash chromatography on silica gel (J. T. Baker, Deventer, Holland) using the equipment described recently (Hofmann and Schieberle, 1995). After the column was flushed with *n*-pentane/diethyl ether (95:5 by vol.; 200 mL), the target compound was eluted with *n*-pentane/diethyl ether (9:1 by vol.; 200 mL).

*[$^2$H]$_2$-(E)-5-Methyl-(E)-2-hepten-4-one.* [$^2$H]$_2$-MHO was obtained by oxidation of [$^2H_2$]-5-methyl-(*E*)-2-hepten-4-ol with pyridinium chlorochromate in dichloromethane following the procedure described above. The reaction mixture was concentrated and separated by flash chromatography using *n*-pentane (150 mL) followed by *n*-pentane/diethyl ether (150 mL; 95:5 by vol.) to isolate the [$^2$H]$_2$-MHO. The concentration was determined using methyl octanoate as the internal standard and using an FID response factor obtained from a mixture containing unlabeled MHO and methyl octanoate (100 μg/mL each).

**Isolation of the Oil from Deshelled Hazelnuts.** Raw hazelnuts (200 g) were frozen in liquid nitrogen then ground in a blender and stirred for 10 min with petroleum benzine (300 mL). After filtration, the retentate was extracted again with petroleum benzine (300 mL). The combined organic layers were then freed from solvent in vacuo. The oil content of the deshelled hazelnuts was 63%.

**Roasting of Hazelnuts.** Fresh hazelnuts (300 g) were thermally treated at about 180 °C for 9 or 15 min, respectively, in a preheated iron pan with continuous stirring. As examined by a panel of 15 trained persons, nuts roasted for 9 min gave the full nutty-roasty flavor, while the aroma of the sample roasted for 15 min was described as burnt.

**Quantification of MHO. Isolation and Enrichment by Extraction and High Vacuum Distillation.** Nuts, either unroasted or roasted, were frozen in liquid nitrogen and then ground in a commercial blender. For quantification, either 0.5 μg of [$^2$H]$_2$-MHO was added to 200 g of the unroasted nut material (or 200 g of the commercial oil from unroasted nuts) or 6 μg of [$^2$H]$_2$-MHO was added to 25 g of the roasted nut material (or 50 mL of oil from roasted nuts). After diethyl ether was added (total volume: 650 mL unroasted material; 100 mL for roasted nuts) and the solution stirred for 30 min at room temperature for equilibration, the solution was filtered.

The volatile fraction and the internal standard were isolated by high vacuum distillation (Guth and Grosch, 1989), and the distillate was treated with aqueous sodium bicarbonate (100 mL; 0.5 mol/L) to remove acidic volatiles. The organic phase was then washed with brine, dried over anhydrous sodium sulfate, and finally concentrated to 1 mL (Schieberle, 1991). MHO and the internal standard [$^2$H]$_2$-MHO were enriched using flash chromatography on silica gel (20 g, column size, 25 cm × 1.5 cm; flow rate, 5 mL/min) as described recently (Hofmann and Schieberle, 1995). After flushing the column with *n*-pentane (150 mL), the target compounds were isolated with *n*-pentane/diethyl ether (150 mL; 95:5 by vol.). The eluate was concentrated to 200 μL by microdistillation (Schieberle, 1991) for mass chromatography.

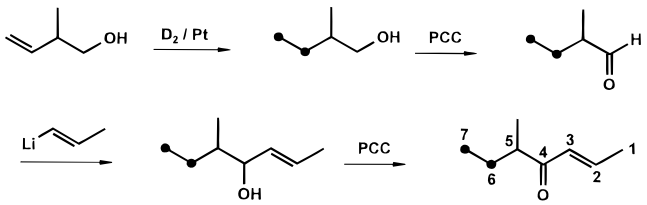


**Figure 1.** Reaction sequence used in the synthesis of [$^2$H]$_2$-5-methyl-(*E*)-2-hepten-4-one [$^2$H]$_2$-MHO (●, deuterium label).

**Simultaneous Steam Distillation/Extraction.** The dried, defatted nut powder was suspended in tap water (200 mL; pH 7.0) and, after addition of [$^2$H]$_2$-MHO (2.5 μg), continuously steam-distilled and extracted with diethyl ether (100 mL) for 1 h in an apparatus according to Likens and Nickerson (cf. Schieberle, 1995a).

**High-Resolution Gas Chromatography (HRGC) Mass Chromatography (MC).** HRGC was performed by means of a Carlo Erba gas chromatograph, type 5160 (Carlo Erba, Hofheim, Germany) using a DB-5 fused silica capillary column (30 m × 0.32 mm; film thickness 0.25 μ; J & W Scientific, Fisons Instruments, Mainz, Germany). After application of the sample (0.5 μL) by the cold on-column injection technique at 35 °C, the temperature was held for 1 min at 35 °C, then raised at 40 °C/min to 60 °C, held isothermally for 1 min, and then raised at 6 °C/min to 230 °C. The flow rate of the carrier gas helium was 2.5 mL/min. HRGC-MC analyses were performed by means of an ion trap mass spectrometer (ITD 800; Finnigan, Bremen, Germany) running in the chemical ionization mode and using methanol as the reactant gas. Mass spectra in the electron impact mode (MS/EI) were generated at 70 eV. Mass chromatograms of the ions *m/z* 127 ($M^+ + 1$: unlabeled MHO) and *m/z* 129−130 ($M^+ + 1$ and $M^+ + 2$; [$^2$H]$_2$-MHO) were recorded, and their relative abundances were calculated by the computer of the mass spectrometer.

A standard curve was generated by analyzing mixtures containing certain amounts of MHO and [$^2$H]$_2$-MHO (Sen et al., 1991). The response factor used was determined to be 0.89 due to the presence of further isotopomers in the labeled MHO which were not used for quantitation (cf. Figure 2).

## RESULTS AND DISCUSSION

**Development of the Isotope Dilution Assay.** The first step in the development of a stable isotope dilution assay is the synthesis of the appropriate labeled isotopomer. As summarized in Figure 1, we followed up a four step route to prepare the labeled [$^2$H]$_2$-5-methyl-(*E*)-2-hepten-4-one. Labeling at positions 6 and 7 was proposed in order to minimize the possibility of proton/deuterium exchange which might be expected in positions 1, 2, 3 and 5. In a first step, the label was, therefore, introduced into 2-methyl-3-buten-1-ol. Oxidation of the alcohol into the corresponding aldehyde, followed by a condensation with propenyl-lithium, generated the [$^2$H]$_2$-5-methyl-(*E*)-2-hepten-4-ol which was finally oxidized to yield the target compound. Analysis of the labeled MHO by MS/EI gave a weak molecular ion at *m/z* 128 (Figure 2b), which was in agreement with the expected incorporation of two deuterium atoms in the target molecule (cf. Figure 2a,b). The fragment *m/z* 69 (Figure 2b), which was in agreement with the spectrum of the unlabeled MHO, on one hand confirmed the presence of the O≡C−CH=CH−$CH_3$ fragment in the synthesized standard, and also showed that no deuterium label was incorporated in positions 1 to 3 of the molecule. Analysis by MS/CI, using methanol as the reagent gas, gave intense ions at *m/z* 129 and 130 (Figure 3), confirming the incorporation of mainly two and, to a lesser extent, three deuterium atoms.

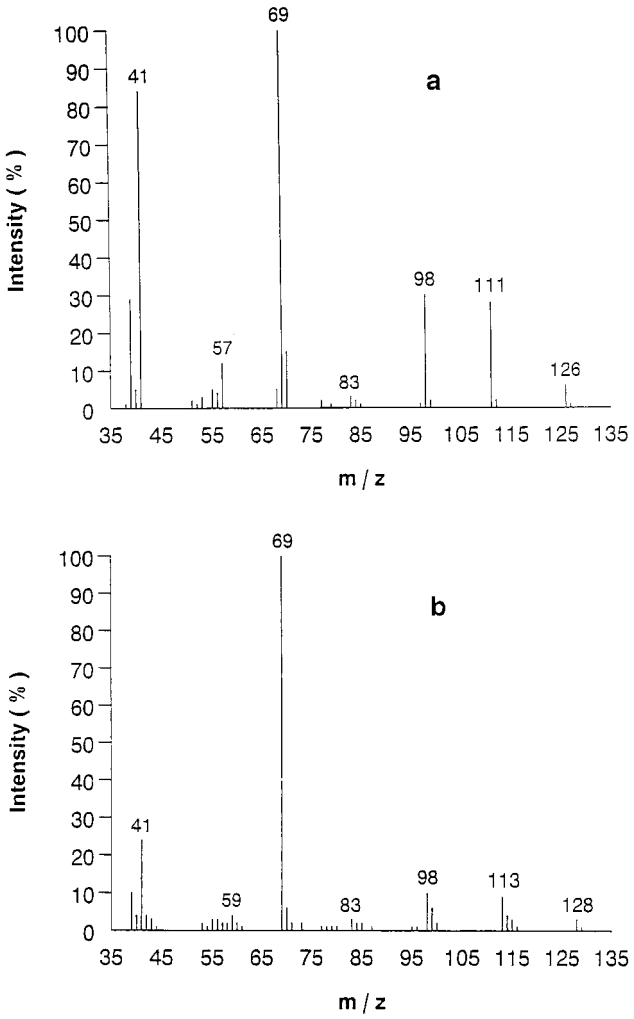


**Figure 2.** Mass spectra (MS/EI) of (a) 5-methyl-(*E*)-2-hepten-4-one and (b) $[^2H]_2$-5-methyl-(*E*)-2-hepten-4-one.

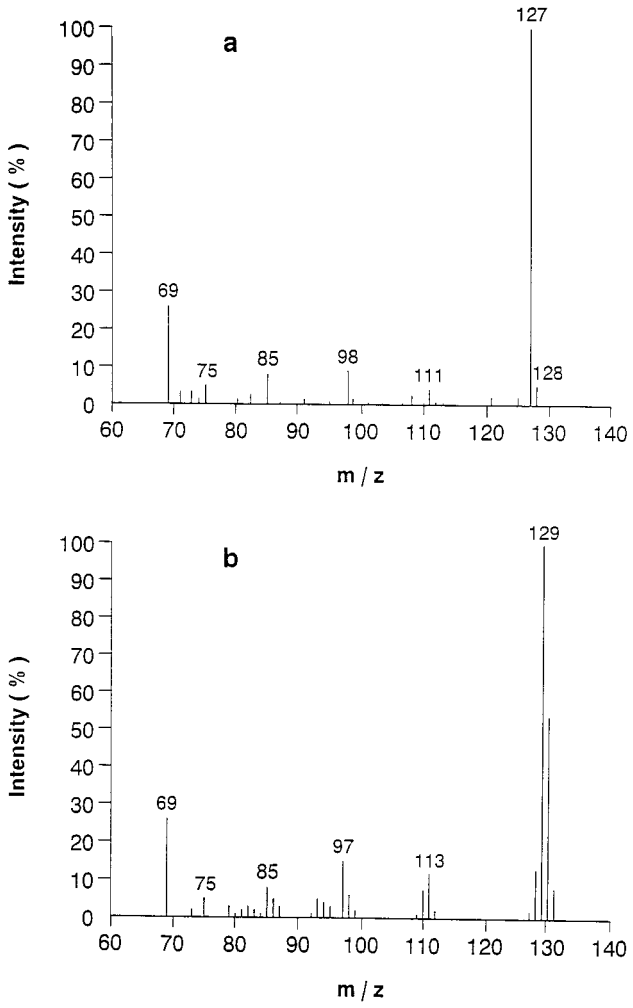


**Figure 3.** Mass spectra of (a) 5-methyl-(*E*)-2-hepten-4-one and (b) $[^2H]_2$-5-methyl-(*E*)-2-hepten-4-one obtained by chemical ionization.

Five mixtures containing known amounts of MHO and the labeled MHO (molar ratios: 5:1 to 1:5) were then analyzed by MS/CI and an average response factor of 0.89 was calculated following the method described by Sen et al. (1991). As an example, the mass chromatogram obtained for a 1:1 mixture of MHO and $[^2H]_2$-MHO (5 ng each) is displayed in Figure 4.

To check whether a deuterium/protium exchange may take place during the workup procedure, a sample of sunflower oil was spiked with the reference aroma compound and the labeled standard (10 $\mu$g/kg each), and the volatile fraction was isolated by high vacuum distillation as described for the nut materials. The value, calculated for MHO on the basis of the amount of the standard added, differed to not more than 5%, thereby corroborating that a deuterium/protium did not interfere with the analytical procedure (data not shown).

**Quantitation of MHO.** In a first experiment, the amounts of MHO in two commercial samples of hazelnut oils processed from either roasted and unroasted nuts were determined. In an oil from unroasted nuts, comparatively low amounts of MHO were present (Table 1). In the oil from the roasted nuts, however, a 49-fold higher amount of MHO was present. Low amounts of MHO were also determined in a self-prepared oil which had been isolated from unroasted hazelnuts. The data indicated the importance of a heat treatment in the generation of this typical hazelnut flavor compound.

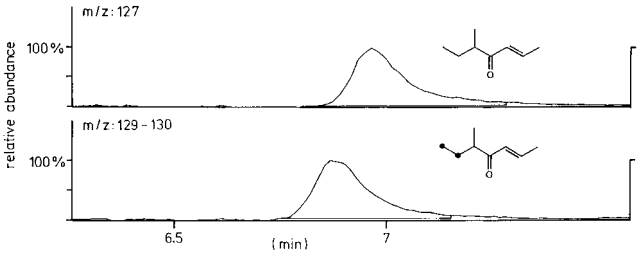


**Figure 4.** Mass chromatograms of a 1:1 mixture of MHO (*m/z* 127) and $[^2H]_2$-MHO (*m/z* 129−130).

**Table 1. Concentrations of 5-Methyl-(*E*)-2-hepten-4-one (MHO) in Hazelnut Samples**

| oil sample | MHO ($\mu$g/kg oil)[a] |
|---|---|
| commercial oil from unroasted nuts | 6.4 |
| commercial oil from roasted nuts | 315.8 |
| self-prepared oil from unroasted nuts | 1.2 |
| boiled, defatted hazelnut powder | 1410[b] |

[a] Data are mean values of triplicates. [b] A sample (10 g) of defatted hazelnut powder isolated from unroasted hazelnuts was continuously steam distilled and extracted for 1 h. Data are based on the dry weight of the defatted powder.

Using steam distillation to isolate the volatiles, Silberzahn (1988) reported much higher concentrations of MHO in unroasted nuts. To elucidate the effect of the SDE procedure on the generation of MHO from the nut

**Table 2. Influence of the Processing Time on the Concentrations of 5-Methyl-(*E*)-2-hepten-4-one (MHO) in Hazelnuts**

| expt | time (min) | MHO ($\mu$g/kg of hazelnuts)[a] |
|---|---|---|
| 1 | 0 | 1.4 |
| 2 | 9 | 661 |
| 3 | 15 | 1148 |

[a] Mean values of triplicates.

material, defatted hazelnut powder from unroasted nuts containing no MHO (<0.1 $\mu$g/kg) was steam distilled and extracted for 1 h in an SDE equipment. The quantitative data revealed (Table 1) that boiling in water generated significant amounts of MHO. Based on an oil content of 63% of the hazelnuts, and assuming that the MHO liberated during roasting is completely transferred into the oil phase, a concentration of 828 $\mu$g MHO per kg of oil would be present as calculated from the data reported for experiment 4 (Table 1). In contrast to previous results (Silberzahn, 1988), the data clearly indicate that raw, unroasted nuts do not contain high amounts of MHO. However, the results imply that the flavorant is preferentially formed from a precursor compound during thermal treatment even at lower temperatures.

To gain some insight into the time course of the flavor generation, raw hazelnuts were roasted for 9 or 15 min in a pan, and the amounts of MHO formed were compared with those present in the unroasted nuts. The results revealed (Table 2) that the amounts of MHO were significantly increased with increasing the roasting time. For example, compared to the unroasted nuts, a more than 600-fold increase in the concentration of MHO was found in nuts roasted for 9 min. These hazelnuts elicited the typical nutty-roasty odor of roasted hazelnuts. Increasing the thermal treatment to 15 min further increased the MHO concentration. However, the overall odor of this material gave a burnt off-note.

## CONCLUSIONS

The data clearly indicate that unroasted hazelnuts do not contain high amounts of MHO but contain significant amounts of a precursor generating the odorant by a thermal treatment. Silberzahn and Tressl (1993) have recently proposed a pathway for the biosynthesis of the flavor compound in raw hazelnuts. On the basis of labeling experiments, the authors evidenced an important role of 2-methylbutanoic acid in the biochemical formation of MHO. The data of our investigations, however, point out that the "thermal" pathway generating MHO from a precursor in the nuts seems to be more effective than the biochemical pathway.

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