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Production of isotopically-labeled standards from a uniformly labeled precursor for quantitative volatile metabolomic studies

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Abstract

Optimal accuracy and precision in small molecule profiling by mass spectrometry generally requires isotopically labeled standards chemically representative of all compounds of interest. However, preparation of mixed standards from commercially available pure compounds is often prohibitively expensive and time consuming, and many labeled compounds are not available in pure form. We used a single prototype uniformly labeled [U-¹³C]-compound to generate [U-¹³C]-volatile standards for use in subsequent experimental profiling studies. [U-¹³C]-α-linolenic acid (C18:3n-3, ALA) was thermally oxidized to produce labeled lipid degradation volatiles which were subsequently characterized qualitatively and quantitatively. Twenty-five [U-¹³C]-labeled volatiles were identified by headspace solid-phase microextraction-gas chromatography-time of flight-mass spectrometry (HS-SPME-GC-TOF-MS) by comparison of spectra with unlabeled volatiles. Using 250 μL starting sample, labeled volatiles were quantified by a reverse isotope dilution procedure. Using the [U-¹³C]-labeled standards, limits of detection comparable to or better than previous HS-SPME reports were achieved, 0.010–1.04 ng/g. The performance of the [U-¹³C]-volatile standards was evaluated using a commodity soybean oil (CSO) oxidized at 60°C from 0 to 15 d. Relative responses of n-decane, an unlabeled internal standard otherwise absent from the mixture, and [U-¹³C]-oxidation products changed by up to 8-fold as the CSO matrix was oxidized, demonstrating that reliance on a single standard in volatile profiling studies yields inaccurate results due to changing matrix effects. The [U-¹³C]-standard mixture was used to quantify 25 volatiles in oxidized CSO and low-ALA soybean oil with an average relative standard deviation of 8.5%. Extension of this approach to other labeled substrates, e.g., [U-¹³C]-sugars and amino acids, for profiling studies should be feasible and can dramatically improve quantitative results compared to use of a single standard.

Keywords

Stable isotope metabolic labeling; lipids; uniformly labeled standard; soybean oil; metabolomics; lipidomics

INTRODUCTION

Stable isotope dilution analysis (SIDA) is commonly employed in quantitative analysis of small molecules by mass spectrometry (MS) to compensate for analytical variability introduced by losses during sample preparation, analyte-specific variation in MS response,

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ASSOCIATED CONTENT

Supporting information Available: Figures S-1 to S-22 and Tables S-1 to S-3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

and general matrix effects such as ion suppression. Typically, individual isotopically labeled analogues for target compounds are combined into a standard mixture for addition to samples. However, in metabolomic and related profiling studies the large number of metabolites, some with unknown structures, renders this approach impractical due to its prohibitive cost and time requirements.¹ One recent approach to overcome this challenge is 'metabolic labeling', in which microorganisms²⁻⁵ or plants⁶⁻⁸ are grown on isotopically labeled substrates (e.g., $^{15}\text{NO}_3^{2-}$, $^{13}\text{CO}_2$) to obtain mixtures of uniformly labeled metabolites. This approach is an extension of previous reports of metabolic labeling in microorganisms, plants, and mammals for SIDA in proteomic experiments.⁹

A stable isotope labeling approach for complex abiotic systems analogous to metabolic labeling has not been presented. Such an approach may be expected to improve accuracy and precision in profiling studies of volatiles in foodstuffs and fragrances by gas chromatography - mass spectrometry (GC-MS), where a single analysis typically detects hundreds of compounds across many chemical classes.¹⁰ SIDA is recommended for quantitative analyses of food and fragrance volatiles due to the low analyte concentrations, often sub-part per billion (ng/g), and potential for sample losses during pre-concentration.¹¹ This is of particular importance when headspace solid-phase microextraction (HS-SPME) or related sorptive techniques are employed. In HS-SPME, volatiles in the headspace are absorbed onto a coated fiber in an inherently chemically-selective and non-quantitative manner and then directly desorbed into the GC injector.¹² As a result, HS-SPME is more susceptible to matrix effects than traditional volatile extraction methods such as liquid-liquid extraction.¹³

A specific example in which a labeling approach would be useful in volatile analysis studies is in studies of edible oil oxidation. More than 70 volatiles have been identified in oxidized oils, including alcohols, aldehydes, ketones, short-chain hydrocarbons, furanones, lactones and fatty acids.¹⁴ These compounds are responsible for 'rancid oil' off-aromas and may also have adverse health effects.¹⁵ HS-SPME is increasingly used for analysis of oil oxidation volatiles as it avoids the tediousness of classic approaches such as simultaneous distillation-extraction. Due to the large number of volatile species produced, preparing an isotopically labeled standard for every volatile species found in oxidized edible oils is cumbersome and expensive. In principle, any variability introduced by the matrix can still be addressed by, for instance, the method of standard addition, but in practice it is common to employ a single non-native internal standard (e.g., *n*-decane)¹⁶ or no internal standard at all.¹⁷⁻¹⁹ This approach may be expected to yield inaccurate results in cases where the oil matrix composition changes from treatment to treatment, e.g., over the time course of oxidation.

We have adapted the stable isotope metabolic labeling strategy to the analysis of volatiles produced by autoxidation of oil. Uniformly labeled α -linolenic acid (*all-Z*-9, 12, 15-C18:3, [U- ^{13}C -ALA]) was intentionally degraded under oxidizing conditions to yield a broad range of [U- ^{13}C]-labeled oxidation products, which were identified and calibrated. The mixture was then used for SIDA of volatiles in edible oil samples. This approach to generating labeled standards for SIDA of volatiles is simple, relatively inexpensive, and does not require prior knowledge of the structure of the analytes. Researchers wishing to apply a similar standard to volatile studies should simply degrade a uniformly labeled precursor and calibrate their instrument accordingly.

EXPERIMENTAL SECTION

Chemical Reagents and Standards

Hexadecane, light and heavy mineral oil, three types of polyethylene glycol (PEG 200, PEG 300 and PEG 400), polypropylene glycol, silicone oil, 2-propanone, 2-propanal, butanal, 1-

penten-3-one, 2,3-pentanedione, hexanal, (E)-3-penten-2-one, (E)-2-pentenal, 1-hydroxy-2-propanone, (E)-2-penten-1-ol, acetic acid, (E,E)-2,4-heptadienal, propanoic acid and 2(5H)-furanone) were purchased from Sigma-Aldrich (St Louis, MO). Propanal, 2-ethylfuran and 2-pentanone were acquired from Acros Organics (Morris Plains, NJ), and acetaldehyde was purchased from Fisher Scientific (Fair Lawn, NJ). A non-labeled ALA standard was obtained from Matreya (Pleasant Gap, PA; 99%). The isotopically labeled ALA standard (99%) was obtained from Cambridge Isotope Laboratories (Andover, MA). The alkane standard (C7-C30) was purchased from Supelco (Bellefonte, PA). Low-ALA soybean oil (LSO) was a generous gift from John Jansen (Bunge, Bradley, IL). A commodity soybean oil (CSO) was acquired from a local supermarket (Ithaca, NY).

HS-SPME-GC-TOF-MS Analyses of Oil Volatiles

A thawed oil sample was added to a 20 mL amber SPME vial along with internal standards (oxidized [U-¹³C]-ALA standard and/or *n*-decane standard solution, both in PEG 400). Volatiles were extracted by HS-SPME using a LEAP CombiPAL autosampler (Carrboro, NC). Samples were incubated for 10 min at an agitation rate of 300 rpm and with an incubation temperature of 50°C prior to fiber insertion. A 2 cm 50/30 μm DVB/CAR/PDMS SPME fiber (Supelco, Bellefonte, PA) was then introduced into the HS and the vial was agitated at 100 rpm for 20 min at 50°C as described by Beltran et al.²⁰

Following HS-SPME, volatiles were thermally desorbed from the fiber into the injector of an Agilent 6890 gas chromatograph coupled to a time-of-flight mass spectrometer (GC-TOF-MS, Pegasus 4D, LECO Corp., St. Joseph, MI). SPME injections were splitless with 5 min of desorption at 250°C. The GC column was a DB-FFAP capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Agilent Technologies Inc., New Castle, DE). The column temperature program was as follows: initial hold for 3 min at 40°C, followed by a 5°C/min ramp to 185°C and then, 8°C/min ramp to 240°C, 5 min hold. Helium was the carrier gas at a flow rate of 1 mL/min, and the detector temperature was 200°C. The TOF-MS was operated in electron impact mode with ionization energy of 70 eV. The electron multiplier was set to 1700 V. MS data were stored at an effective acquisition rate of 5 spectra/s over a mass range of *m/z* 35–400, and data processing was carried out by the native LECO ChromaTOF software.

Optimization of ALA Oxidation Conditions and Characterization of Products

To select a solvent for ALA oxidation that would yield minimal interferences, 500 μL of LSO was mixed with 500 μL of various test solvents and analyzed using the HS-SPME-GC-TOF-MS conditions described above. The solvents evaluated were hexadecane, light mineral oil, heavy mineral oil, silicone oil, PEG 200, PEG 300, PEG 400 and polypropylene glycol, all chosen for their relative stability to oxidizing conditions.

Unlabeled ALA standard was oxidized based on the protocol described in the AOCS Oven Storage Test Cg 5-97.²¹ One hundred mg of ALA were dissolved in 10 g of PEG 400 in an amber 20 mL SPME vial. The vial was sparged with O₂ for 5 min, sealed with Teflon and vortexed. The sealed vial was then incubated at 60°C in the dark. After 72h of oxidation, the oxidized sample was analyzed by the same HS-SPME-GC-TOF-MS method to characterize ALA degradation products. A total of 25 volatile compounds were identified by comparison of mass spectra and Kovats retention indexes (RI) to authentic standards when available. In the absence of authentic standards, tentative identification was based on comparison of mass spectra to the 2005 NIST library database (similarity > 85%) and RI. To calculate the non-isothermal RI,²² an alkane standard (C7-C30) dissolved into dimethyl sulfoxide was analyzed following the same experimental conditions.

To optimize the oxidation time, an analogous solution of ALA in PEG 400 (10 mg/g) was placed in an amber 20 mL SPME vial with O₂ in the headspace and incubated at 60°C in the dark. Aliquots of the degraded standard (100 µL) were taken after 0, 1, 2, 6, 7, 9, 10 and 16 d of oxidation and added to 1 mL of LSO. Samples were sparged with N₂ and stored at –80°C until analysis. A 250 µL sub-sample of this mixture was then analyzed following the above HS-SPME-GC-TOF-MS methodology. Analyses were performed in triplicate on the same degraded sample.

Production of Oxidized [U-¹³C]-ALA Standard and Characterization of [U-¹³C]-Volatiles

[U-¹³C]-ALA (100 mg) was dissolved in 10 g of PEG 400 in an amber 20 mL SPME vial, sparged with O₂ for 5 min, sealed with Teflon and vortexed. The sealed vial was incubated at 60°C in the dark for 7 d. The solution was then dispensed into 2 mL amber vials and kept at –80°C with N₂ in the headspace until analysis.

To characterize the [U-¹³C]-volatile compounds, the oxidized ALA standard, the oxidized [U-¹³C]-ALA standard and a 50:50 solution of both mixtures were analyzed by HS-SPME-GC-TOF-MS under the conditions described above. Identification of the [U-¹³C]-analogues of the 25 non-labeled compounds characterized from ALA oxidation was accomplished by comparison of retention times and observation of an appropriate mass shift between their mass spectra.

Evaluating Stability of ALA Degradation Products

To study the stability of the ALA degraded standard, 100 mg of ALA was added to an amber 20 mL SPME vial and dissolved in 10 g of PEG 400. The vial was sparged with O₂ for 5 min, sealed with Teflon and vortexed. The sealed vial was then incubated at 60°C in the dark. After 7 d of oxidation, 500 µL of the solution was aliquoted into 2 mL amber vials with N₂ in the headspace and stored at one of 6 temperatures (–80°C, –20°C, +2°C, +13°C, +20°C and +60°C). The stability of the oxidized mixture was evaluated after 2 and 4 weeks of storage by comparing the relative peak heights (unlabeled analyte/[U-¹³C]-analyte) measured by HS-SPME-GC-TOF-MS.

Linearity and Limits of Detection of Method

Serial dilutions of eighteen of the unlabeled volatiles in PEG 400 (20 µL) were added to 180 µL of LSO oil, and spiked with 30 µL of the oxidized [U-¹³C]-ALA standard and 20 µL of the *n*-decane standard solution (0.01 mg *n*-decane in 1 g of PEG 400) prior to analysis. Calibration curves consisted of the peak height ratio of the analyte to the respective [U-¹³C]-standard²³ plotted against the known concentrations of the unlabeled compounds. LOD were calculated following the method of Pallesen.²⁴

HS-SPME Analyses of Oil Oxidation Volatiles: Comparison of Performance of *n*-Decane and [U-¹³C]-Internal Standards

Thawed CSO samples (200 µL) at different degradation times (*t* = 0, 1, 2, 3, 6, 7, 9, 12 and 15 d) were spiked with 30 µL of the oxidized [U-¹³C]-ALA standard and 20 µL of the *n*-decane standard solution (0.01 mg *n*-decane in 1 g of PEG 400) in an amber 20 mL SPME vial. Volatiles were analyzed in triplicate on the same degraded sample by the HS-SPME-GC-TOF-MS method described above. The concentrations of the 25 volatiles derived from ALA oxidation in CSO samples were determined by interpolation on both calibration curves (i.e., [U-¹³C] and *n*-decane). For each analyte, the ratio $R_{13C,d} = [\text{analyte}]_{U-13C} / [\text{analyte}]_{n\text{-decane}}$ was calculated, where the numerator is the concentration estimated with respect to the *n*-decane internal standard and the denominator is the concentration estimated with respect to the [U-¹³C]-internal standard.

Analysis of LSO and CSO Samples

LSO and CSO were oxidized by a standard procedure for evaluating oil stability.²¹ Ten mL of oil were placed in an amber 20 mL SPME vial, sparged with O₂ for 5 min and sealed with a Teflon-lined cap. Oils were incubated at 60°C in the dark. After 15 d of storage, the headspace of the container was sparged with N₂ and 250 µL of the sample were analyzed in triplicate before (t=0 d) and after oxidation (t=15 d) under identical HS-SPME-GC-TOF-MS conditions.

FA compositions of LSO and CSO prior to oxidation were determined by the one step extraction/methylation method described elsewhere.²⁵ Analyses were performed in triplicate on an HP 5890 Series II gas chromatograph coupled to a flame ionization detector (GC-FID) (Hewlett Packard, Palo Alto, CA) equipped with a BPX70 fused-silica capillary column (25m × 0.22 mm i.d. × 0.25 µm film thickness; SGE Inc., Austin, TX). The column temperature program was as follows: the initial temperature of 80°C was ramped up to 170°C at 30°C/min, 2 min hold, then increased to 240°C at 10°C/min, 14 min hold. The injector was at 250°C in splitless mode and hydrogen was used as carrier gas at a flow rate of 1mL/min.

Statistical Analysis

Calibration curves and statistical analyses were conducted with JMP Version 9 (SAS Institute, Cary, NC). Paired comparisons, using Student's *t* test, were used to evaluate the thermal stability of the ALA degradation products and to compare the ratios R_{13C,d} at different time points. *P* < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

HS-SPME-GC-TOF-MS Analyses of Oil Volatiles

The HS-SPME and GC conditions were adopted from previous studies without modification. A 2 cm 50/30 µm DVB/CAR/PDMS fiber was selected based on previous oil volatile studies that compared fiber types.^{26, 27} This three-phase SPME fiber shows maximum absorption for the middle volatility range of compounds²⁸ and can achieve between day precision of <10%.²⁹ Extraction time and temperature were based on the optimized conditions described by Beltran et al.²⁰ The GC gradient temperature program was adapted from Jelen et al.²⁶

Optimization of ALA Oxidation and Characterization of Products

The initial goal of our work was to produce a [U-¹³C]-standard mixture appropriate for oil volatile analyses by degradation of [U-¹³C]-ALA. Because we wished to degrade the [U-¹³C]-ALA quantitatively, it was necessary to first identify an inert solvent for the [U-¹³C]-ALA so that any evolved volatiles would be solubilized and effectively captured. Hexadecane, light mineral oil, heavy mineral oil, silicone oil, PEG 200, PEG 300, PEG 400 and polypropylene glycol were all tested because they were expected to be thermally stable, have low vapor pressures, and be miscible with ALA and oil samples. The tests were performed by dissolving 500 µL of LSO in 500 µL of each inert solvent. HS-SPME and GC analysis lead to our choice of PEG 400 because it yielded the fewest volatile compounds detectable by SPME-GC-TOF-MS (data not shown). In total, 25 volatiles (listed in Table 1) derived from the oxidation of unlabeled ALA in PEG 400 (10 mg/g) during accelerated storage conditions were characterized by HS-SPME-GC-TOF-MS. Their mass spectra and RI were compared to those from authentic standards when available. If the appropriate standard was not available, tentative identification was based on comparison of mass spectra to the NIST library (similarity>85%) and RI. The ALA-derived compounds have been

previously identified in studies of oxidation of n-3 fatty acid standards,³⁰ as well as n-3 rich foodstuffs including anchovies,³¹ fish oil-enriched milk emulsions³² and oils.³³

To determine the optimal incubation time for maximizing the concentrations of the target volatiles, an unlabeled ALA standard was subjected to the oven storage test for varying time periods.²¹ The test was performed at 60°C because this temperature was used in an AOCS collaborative study³⁴ and is commonly employed in oil oxidation research.^{26, 35, 36} A solution of unlabeled ALA in 400 PEG (10 mg/g) was placed in an oven at 60°C for 0, 1, 2, 6, 7, 9, 10 and 16 d in the dark with O₂ in the headspace. Responses for 16 volatiles from ALA degradation were monitored, and time courses for 6 representative volatiles are shown in Figure 1. Propanal and (E,E)-2,4-heptadienal, both characteristic compounds of ALA oxidation,³⁰ increased rapidly during the first week of oxidation before decreasing. In contrast, volatiles such as 2-ethylfuran, 2-pentanone, 1-penten-3-one, 1-penten-3-ol, 3-hexen-2-one, (E)-2-penten-1-ol, 2-hydroxy-3-pentanone, 1-hydroxy-2-butanone or 5-ethyl-2(5H)-furanone increased throughout the whole experimental period. (E)-2-butenal, (E)-3-penten-2-one and (E)-2-pentenal initially increased and reached a plateau after 6 d of oxidation. Based on these results, a 7 d oxidation period was selected to yield a broadly representative profile of ALA oxidation products (Figure 1).

Production of Oxidized [U-¹³C]-ALA Standard and Characterization of [U-¹³C]-Volatiles

Once optimal oxidation conditions were established, unlabeled ALA and [U-¹³C]-ALA standards were heated in PEG 400 for 7 d at 60°C. All 25 target labeled compounds could be identified in the [U-¹³C]-standard, with identifications based on the presence of an appropriate mass shift of their mass spectra in comparison to the respective unlabeled volatile. As an example, Figure 2 shows the partial chromatograms and the mass spectra of three ALA degradation metabolites (propanal, 2-ethylfuran and (E)-2-pentenal) and their corresponding [U-¹³C]-analogues. The [U-¹³C]-propanal mass spectrum has prominent ions at *m/z* 60 and 61 (*M*⁺11) three amu higher than the respective ions *m/z* 57 and 58 (*M*⁺12) from unlabeled propanal. A similar shift was also detected for *M*⁺ in unlabeled 2-ethylfuran and [U-¹³C]-2-ethylfuran (*m/z* 96 and 102, respectively) and for unlabeled (E)-2-pentenal and [U-¹³C]-(E)-2-pentenal (*m/z* 84 and 89, respectively). The present stable isotope metabolic labeling could be considered successful by comparison of the mass spectra of the 25 unlabeled vs [U-¹³C]-labeled compounds identified in the current manuscript (see Supporting Information, Figures S-1 to S-22).

The concentrations of the 25 [U-¹³C]-volatiles in the standard mixture were quantified by interpolating the response of a labeled standard to a calibration curve prepared by increasing amounts of unlabeled analogues to the labeled standard mixture, a procedure referred elsewhere as “reverse isotope dilution”⁸ which was previously used to quantify [U-¹³C]-metabolites in yeast.³⁷ The concentrations are presented in Table 1. On a w/w basis, the major volatile produced was acetic acid, followed by (E,E)-2,4-heptadienal, propanoic acid, 1-penten-3-ol, acetaldehyde, and propanal.

Evaluating Stability of ALA Degradation Products

Many of the products of ALA oxidation (ketones, dicarbonyls, unsaturated hydrocarbons) are potentially reactive, and as such may not be stable if stored as a mixture. To study the stability of the ALA degraded standard, a 7 d oxidized ALA standard was stored under N₂ at different temperatures (−80°C, −20°C, +2°C, +13°C, +20°C and +60°C) for either 2 or 4 weeks. There were no significant differences in the ALA-derived volatiles for the samples stored at −80°C and −20°C after one month of storage (see Supporting Information, Table S-1) and minimal differences (n.d. to 15%) were observed at the +2°C storage temperature. Significant changes were observed in several volatiles after 1 month at storage temperatures

> +2°C, especially for 2-pentanone, 1-penten-3-one and 1-penten-3-ol. Thus, we expect that the [U-¹³C]-ALA derived standard mixture should be stable under suitable storage conditions, i.e., in the freezer and with N₂ in the headspace. However, the poor stability of the standard at temperatures > +2°C, suggests that care should be taken to use the standard quickly once thawed to avoid changes in its volatile composition.

Linearity and Limits of Detection of Method

Eighteen compounds of various retention times covering analytes with a range of polarities and volatilities (see Supporting Information, Table S-2) were selected across the entire GC chromatogram for evaluation of figures of merit. Since neither 5-methyl-2(5H)-furanone nor 5-ethyl-2(5H)-furanone were commercially available, concentrations of these compounds were expressed as 2(5H)-furanone equivalents.

Table S-2 (see Supporting Information) displays retention indexes, linear ranges, limits of detection (LOD) and quantifier and qualifier ions for these 18 volatiles. Good linearity ($R^2 = 0.9819 - 0.9998$) and LOD between 0.010 and 1.045 ng/g were achieved for all volatiles. A linear range of at least an order of magnitude was achieved for all volatiles, and a linear range of >2 orders of magnitude was observed for 12 of the 18 volatiles. Propanal had the lowest LOD (0.01 ng/g), an improvement of more than one order of magnitude in comparison with previous values,^{38, 39} and its response was linear over the entire concentration range examined (0.125 – 10000 ng/g; $R^2 = 0.9971$; see Supporting Information, Table S-2).

The variation observed in the linear ranges reported in Table S-2 (see Supporting Information) is likely due in part to the varying concentrations of [U-¹³C]-labeled volatiles in the oxidized standard (Table 1). Labeled volatiles that were at low concentrations in the standard mixture (e.g., [U-¹³C]-2,3-pentanedione) tended to have more limited linear ranges. This limitation could be overcome by adding a larger amount of [U-¹³C]-internal standard, or else tuning the oxidation protocol to increase production of the target volatiles.

HS-SPME Analyses of Oil Oxidation Volatiles: Comparison of Performance of *n*-Decane and [U-¹³C]-Internal Standards

HS-SPME lipid oxidation analyses are frequently performed with a single non-labeled internal standard.¹⁶ To determine if using a [U-¹³C]-standard mixture would improve the accuracy and/or precision of HS-SPME-GC-TOF-MS measurements as compared to a single unlabeled internal standard, CSO was oxidized by heating samples at 60°C in the presence of O₂. Samples were incubated for 0, 1, 2, 3, 6, 7, 9, 12 and 15 d, and both the [U-¹³C]-standard mixture and a non-labeled *n*-decane internal standard were added prior to analysis. Several non-labeled internal standards have been proposed for studies of lipid oxidation volatiles, including *n*-decane, 2-heptanone, 4-methyl-1-pentanol and 4-heptanone.¹⁶ Based on a preliminary investigation, *n*-decane was selected because it was the best resolved chromatographically under our experimental conditions (data not shown). Chromatographic analyses were carried out in triplicate and the concentration of the 25 target volatiles was determined by interpolation on the appropriate [U-¹³C] or *n*-decane calibration curve. A calibration curve for a compound of similar structure was employed for those analytes that did not have their own calibration curve. Thus, 2-butanone, (E)-2-butenal, 1-penten-3-ol, 3-hexen-2-one, 2-hydroxy-3-pentanone, 1-hydroxy-2-butanone, 5-methyl-2(5H)-furanone and 5-ethyl-2(5H)-furanone were quantified with 2-pentanone, (E)-2-pentenal, (E)-2-penten-1-ol, (E)-3-penten-2-one, 1-hydroxy-2-propanone, 1-hydroxy-2-propanone, 2(5H)-furanone and 2(5H)-furanone calibration curves, respectively.

As expected, the responses of all measured volatiles increased with increasing oil oxidation time (data not shown). The mean relative standard deviation (RSD) was 7.3% using the [U-¹³C]-ALA standard and 11.3% when *n*-decane was employed. This improvement in precision is expected since an isotopically labeled standard should better reflect any differences in extraction efficiency, losses in the injector, and related factors.²³

To determine if the [U-¹³C]-ALA standard yielded improved accuracy with respect to the *n*-decane standard, we calculated the ratio of the responses as $R_{13C,d} = [\text{analyte}]_{U-13C} / [\text{analyte}]_{n\text{-decane}}$. The apparent concentrations for 8 representative compounds are presented in Figure 3. The observed ratios for some volatiles (acetic acid, propanoic acid, acetaldehyde and 2-ethylfuran) did not change significantly as the oil was increasingly oxidized. However, the concentrations of these compounds calculated as *n*-decane equivalents are different than the (presumably) more accurate concentration calculated with respect to the [U-¹³C]-standard. For example, carboxylic acids concentrations in all samples were 4-fold higher when the *n*-decane internal standard was used. Calibration standards were prepared in PEG 400 to avoid further oxidation, and the fact that $R_{13C,d} \approx 1$ likely reflects matrix differences between the calibration curves and real samples. However, because the $R_{13C,d}$ value was constant during the course of oxidation for these compounds, it should be possible to use decane as an internal standard and achieve accurate semi-quantitative data.

In contrast, for other analytes, $R_{13C,d}$ changed significantly as the oil was oxidized. As shown in Figure 3, at an extreme, an eight fold increase in $R_{13C,d}$ of (E)-2-pentenal was observed from day 0 ($R_{13C,d} = 0.04$) to day 15 ($R_{13C,d} = 0.31$). A parallel increase was also observed for $R_{13C,d}$ of (E)-2-butenal, from 0.05 to 0.27. Conversely, the $R_{13C,d}$ of (E,E)-2,4-heptadienal decreased by a factor of 1.6 over the course of oxidation. The $R_{13C,d}$ for propanal also decreased significantly after 6 d of oxidation. The concentrations determined with respect to the [U-¹³C]-calibration curves are expected to be more accurate than the concentrations determined with respect to the *n*-decane standard, but the reasons for the changes in $R_{13C,d}$ are not clear. One possible explanation is that as the oil samples are oxidized and more volatiles are produced, competition on the SPME fiber for binding sites increases. This competition may affect the extraction efficiency of the slightly polar analytes like unsaturated aldehydes more than the non-polar *n*-decane. Additionally, the thermal oxidation of edible oils is expected to change the oil matrix, e.g., through autooxidation, isomerization, and polymerization reactions,¹⁰ which could also potentially alter the volatility and SPME extraction efficiency of the different species.

The variation in $R_{13C,d}$ for several compounds suggests that preparing robust calibration curves with a single standard, e.g., *n*-decane, will be difficult. Accurate quantification could be performed by more tedious methods, e.g., preparing calibration curves for each time-point of oxidation in the different oils to avoid matrix effects, which would be cumbersome for a large number of samples. The use of a [U-¹³C]-ALA labeled standard mixture appears to be a simple solution to account for inconsistent matrix effects.

Analysis of LSO and CSO Samples

As a demonstration of its utility, the degraded [U-¹³C]-ALA standard was used to quantify volatiles during thermal oxidation of two soybean oils differing in ALA concentration. Prior to oxidation, the FA profile of the oils was determined by GC-FID. The composition of the low ALA oil (LSO) was (% of total FA) 16:0 (10.5), 18:0 (5.6), 18:1 (24.3), 18:2 (54.7) and 18:3 n-3 (3.1). LSO is a low-ALA soybean oil which the FA profile has been purposefully altered to increase the storage stability and improve its performance in frying applications. The composition of the higher ALA oil (CSO) was 16:0 (10.9), 18:0 (3.3), 18:1 (24.9), 18:2 (52.0) and 18:3 n-3 (7.2).

Volatiles from LSO and CSO were quantified before ($t = 0$) and after ($t = 15$ d) oxidation at 60°C following the conditions described previously. Table S-3 (see Supporting Information) shows the volatile profile of LSO and CSO during oxidative storage. The $[\text{U-}^{13}\text{C}]$ -standard mixture was used as an internal standard, and the 25 target volatiles were successfully quantified with an average RSD of 8.5%. All volatiles increased significantly after 15 d of oxidation and, as expected, the highest concentrations were detected for CSO. The major volatile compounds observed for both oxidized oils were acetaldehyde, propanal, hexanal, acetic acid, (E,E)-2,4-heptadienal and propanoic acid. The largest differences between LSO and CSO after thermal oxidation were observed for (E)-2-penten-1-ol and 2-ethylfuran, typical degradation products from linolenic acyl groups.^{30, 33} (E,E)-2,4-heptadienal and hexanal were at concentrations higher than the linear range. This problem can be readily overcome by dilution of the sample. In the case of hexanal, which is formed primarily by oxidation of n-6 fatty acids,³³ a better linear range could likely be achieved by inclusion of an oxidized $[\text{U-}^{13}\text{C}]$ -linoleic acid standard, prepared in a similar fashion to our current approach for $[\text{U-}^{13}\text{C}]$ -ALA.

CONCLUSIONS

We have demonstrated that a wide range of $[\text{U-}^{13}\text{C}]$ -labeled compounds can be generated from a $[\text{U-}^{13}\text{C}]$ -ALA substrate for use as internal standards in volatile analyses, overcoming one of the major limitations in quantitative multi-target studies. This approach is analogous to earlier ‘metabolic labeling’ studies applied to biomolecules. The $[\text{U-}^{13}\text{C}]$ -standard is stable at freezer temperatures and under N_2 atmosphere, and it provides advantages in both precision and accuracy when compared to use of a single, non-labeled internal standard. This approach is expected to be broadly useful for analysis of volatiles in foods and fragrances, since many naturally occurring volatiles are produced by degradation or reaction of a limited number of non17 volatile biomolecules (e.g., lipids, amino acids, sugars) and are available commercially in uniformly labeled form.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. HS-SPME-GC-TOF-MS responses for propanal, 1-penten-3-ol, (E)-2-pentenal, 2-ethylfuran, (E,E)-2,4-heptadienal and 5-ethyl-2-(5H)-furanone during ALA oxidation.

Figure 2.

Chromatograms and mass spectra of three representative compounds formed by thermal degradation of α -linolenic acid (ALA) and $[U-^{13}C]$ -ALA standards. (A) propanal and $[U-^{13}C]$ -propanal (quantifier ions 58 and 61, respectively), (B) 2-ethylfuran and $[U-^{13}C]$ -2-ethylfuran (quantifier ions 81 and 86, respectively), and (C) (E)-2-pentenal and $[U-^{13}C]$ -(E)-2-pentenal (quantifier ions 83 and 59, respectively).

Figure 3.

Fold change of the concentrations estimated by HS-SPME-GC-TOF-MS using [U- ^{13}C]-labeled standards vs. *n*-decane for **(A)** acetic acid, **(B)** propanoic acid, **(C)** (E)-2-butenal, **(D)** (E)-2-pentenal, **(E)** (E,E)-2,4-heptadienal, **(F)** acetaldehyde, **(G)** 2-ethylfuran and **(H)** propanal. Data was calculated as $R_{13C,d} = [\text{analyte}]_{\text{U-}^{13}\text{C}}/[\text{analyte}]_{n\text{-decane}}$. Absence of letters indicates no significant differences among time points.

Table 1

Volatile composition of the [U-¹³C]-standard mixture and concentration of each [U-¹³C]-volatile expressed as ng/mg [U-¹³C]-ALA, in order of retention index.

[U- ¹³ C]-Compound	Composition (%)	ng volatile/mg [U- ¹³ C]-ALA (mean ± SD)
Acetaldehyde	8.0	117.4 ± 11.2
Propanal	7.0	102.8 ± 9.0
2-Propanone	0.1	1.4 ± 0.1
2-Propenal	0.2	3.7 ± 0.3
Butanal	0.3	4.2 ± 0.3
2-Butanone	0.004	0.1 ± 0.0
2-Ethylfuran	0.1	1.4 ± 0.2
2-Pentanone	0.1	1.4 ± 0.2
1-Penten-3-one	0.2	2.7 ± 0.4
(E)-2-butenal	1.8	25.7 ± 2.3
2,3-Pentanedione	0.1	1.1 ± 0.1
Hexanal	3.0	44.1 ± 10.9
(E)-3-penten-2-one	0.2	2.8 ± 0.2
(E)-2-pentenal	1.5	22.3 ± 1.6
1-Penten-3-ol	12.2	178.8 ± 14.3
3-Hexen-2-one	0.05	0.7 ± 0.0
1-Hydroxy-2-propanone	0.8	11.9 ± 0.5
(E)-2-penten-1-ol	0.2	3.6 ± 0.1
2-Hydroxy-3-pentanone	0.4	5.5 ± 0.1
1-Hydroxy-2-butanone	1.0	14.2 ± 0.8
Acetic acid	24.7	362.4 ± 16.2
(E,E)-2,4-heptadienal	19.0	277.8 ± 54.3
Propanoic acid	18.1	264.8 ± 15.8
5-Methyl-2(5H)-Furanone	0.3	4.9 ± 0.2
5-Ethyl-2(5H)-Furanone	0.7	9.6 ± 0.3