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Trace quantitation of 4-hydroxy-2-nonenal in biological samples as its oxime–bis-*tert.*-butyldimethylsilyl derivative using 3-hydroxynonanal as an internal standard☆

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ABSTRACT

A gas chromatographic–mass spectrometric method for the determination of the lipid aldehyde 4-hydroxy-2-nonenal (4HNE) in trace quantities is described. The method utilizes the reaction of aldehydes with hydroxylamine leading to the formation of the oxime derivative. The aldehydes are recovered by octadecylsilyl solid-phase extraction and converted to the bis-*tert.*-butyldimethylsilyl derivatives for analysis using electron ionization. A novel 4HNE analogue, 3-hydroxynonanal, has been synthesized and is used as an internal standard. A limit of detection of approximately 1 pmol of 4 HNE in preparations of approximately $2 \cdot 10^6$ cells or 0.5 ml of whole blood, plasma or serum was observed. Standard addition analysis indicates that the method is accurate at these levels. Replicate analysis of the National Institutes of Standards and Technology Standard Reference Material SRM 909 indicates an average in-run precision of 8.1% and a between-run precision of 13.5% at an average concentration of 82.1 pmol/ml of reconstituted material.

INTRODUCTION

4-Hydroxy-2-nonenal (4HNE) is emerging as an important by-product of lipid peroxidation. 4HNE has been shown to be toxic to cultured cells [1,2] and to be able to modify low-density lipoprotein in a manner which appears to promote atherosclerosis [3–5]. At the present time, several methods for the measurement of 4HNE in biological materials have been reported including a high-performance liquid chromatographic method [6] and several gas chromatographic–mass spectrometric (GC–MS) methods [7–11]. Continued development of trace analysis techniques for 4HNE is needed to support further investigation of the formation and actions of this compound.

The method described here employs a novel analogue of 4HNE, 3-hydroxynonanal (HNA), as an internal standard. This compound is a saturated aldehyde with the hydroxyl group in the 3-position. The other internal standards which have been used are a stable isotope-labeled analogue of 4HNE, [2,3-$^2H_2$]4-hydroxy-2-nonenal (d_2-4HNE) [7] and cyclohexanone [9]. In general,

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stable isotope-labeled analogues are ideal internal standards. In this case, however, the stability of the deuterium label is of concern especially in the 3-position at which the sulfhydryl group of glutathione and cysteine attack 4HNE in what has been shown to be an important metabolic pathway [1,12,13]. This conjugation reaction results in a saturated double bond and has been shown to occur both enzymatically and non-enzymatically. Since this reaction does have a reverse component, it would appear possible that the stable isotope label could be lost from both the 2- and 3-positions resulting in the artifactual production of unlabeled or partially labeled 4HNE from the internal standard which is added. Cyclohexanone is not an ideal internal standard because it does not possess all of the functional characteristics of 4HNE; specifically it is a ketone rather than an aldehyde and is not hydroxylated. The result is that both the extraction and derivatization chemistry could be very different for cyclohexanone and 4HNE depending on the chemistry used and the system being studied. A saturated analogue such as HNA would not undergo the sulfhydryl conjugation reaction so it could not be converted to 4HNE, yet still has the aldehyde and hydroxyl functional groups giving it similar solubility and reaction characteristics.

A second possible metabolic reaction of 4HNE is binding to protein through Schiff base linkages. This reaction would not be expected to be enzyme-catalyzed but the large number of free amine residues available on proteins in biological material make this a potentially important pathway. Quantitation of 4HNE in either its free form or bound to protein through Schiff base linkages is achieved by forming an oxime derivative of the aldehyde in a nucleophilic substitution reaction. As shown in Fig. 1, reactions a and b, this type of reaction forms the same product for both the free and Schiff base-bound form releasing the compound from the Schiff base. The bis-*tert.*-butyldimethylsilyl derivative is then formed prior to analysis by selected ion monitoring (SIM) GC–MS. The primary advantage of this derivative for aldehydes is that the facile loss of a *tert.*-butyl moiety from the molecular ion

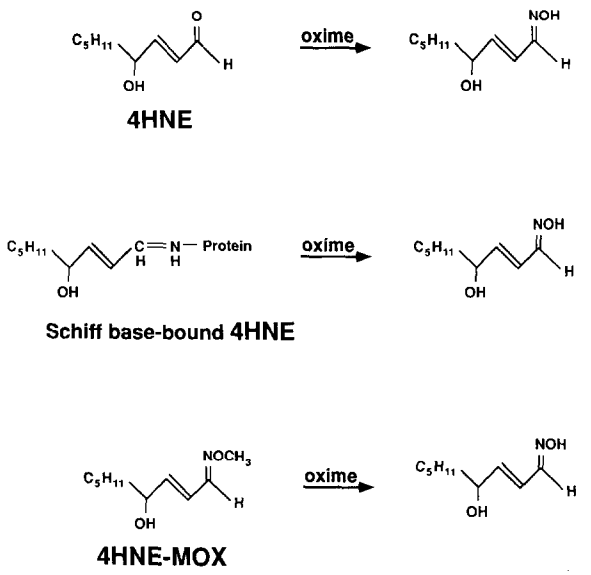

Fig. 1. Reactions of (a) 4HNE, (b) Schiff base-bound 4HNE and (c) 4HNE-MOX to form the oxime derivative of 4HNE.

results in a characteristic $[M - 57]^+$ ion which carries a high proportion of the total ion current. These derivatives are also stable to water hydrolysis, which allows a final extraction to remove the derivatizing reagent from the sample which is injected onto the column.

EXPERIMENTAL

Reagents and standards

All solvents were HPLC grade and purchased from commercial sources. Hydroxylamine hydrochloride (oxime), anhydrous dimethylformamide (DMF) and all reagents used in the synthesis of 4HNE and HNA were purchased from Aldrich (Milwaukee, WI, USA). N-Methyl-N-*tert.*-butyldimethylsilyltrifluoroacetamide (MTBSTFA) was purchased from Pierce (Rockford, IL, USA). The octadecylsilyl solid-phase extraction columns were purchased from J. T. Baker (Phillipsburg, NJ, USA). Standard Reference Material (SRM) 909 was purchased from the National Institute of Standards and Technology (NIST, formerly the National Bureau of Standards, Gaithersburg, MD, USA) and reconstituted according to NIST procedure A.

4HNE was synthesized according to the meth-

od of Esterbauer [14]. Briefly, propiolaldehyde diethyl acetal was converted to an MgBr Grignard by reaction with ethyl magnesium bromide in tetrahydrofuran (THF) at −10°C. Hexanal in THF was added at −10°C to form 4-hydroxy-2-nonynal diethyl acetal which was reduced to 4-hydroxy-2-nonenal diethyl acetal with $LiAlH_4$ in THF at −10°C. The diethyl acetal was hydrolysed in refluxing citric acid to give 4HNE with an overall yield of approximately 75%. The methoxime (MOX) derivative of 4HNE was used for the preparation of low-concentration standards. This derivative was formed by adding appropriate amounts of 4HNE to 0.3 *M* methoxime hydrochloride in 0.1 *M* acetate, pH 5. The 4HNE-MOX was then recovered by extracting the reaction mixture with methylene chloride. Dilute solutions were prepared by evaporating small aliqouts of the methylene chloride solution and reconstituting in methanol. The concentration of these solutions could be determined spectrophotometrically with λ_{max} = 234 nm and ξ = 13 950 l mol^{-1} cm^{-1}.

HNA was synthesized by the reaction of mercuric acetate with 2-nonenal. In our procedure, 40 mmol of 2-nonenal was converted to the ethylene acetal by refluxing in toluene with ethylene glycol using *p*-toluenesulfonic acid as a catalyst. The ethylene acetal solution was washed with 1 *M* NaOH and water and the acetal recovered by evaporating the toluene. This product was placed in a 250 ml round-bottom flask containing 40 ml of THF, and 50 mmol of mercuric acetate in 30 ml of water were added. The reaction proceeded for 30 min at room temperature with stirring. A 50-ml volume of 3 *M* NaOH was added followed immediately by 50 ml of 0.5 *M* $NaBH_4$ in 3 *M* NaOH. The yellow–orange mixture immediately turned grey and the precipitation of mercury was observed. The aqueous solution was decanted off the mercury and extracted with three 60-ml aliquots of light petroleum (b.p. 35–60°C). The organic fractions were washed with saturated NaCl, dried over anhydrous sodium sulfate and evaporated to yield the product. The product was transferred to a 250-ml round-bottom flask containing 150 ml of acetone, and 40 ml of 1 *M* HCl were added. The mixture was heated at vigorous reflux for 15 min and cooled. The acid was then carefully neutralized with 1 *M* $NaHCO_3$ and the mixture extracted with three 50-ml aliquots of methylene chloride. The methylene chloride was dried over anhydrous sodium sulphate and evaporated to yield 6.39 g (40 mmol, 100% yield) of the final product. GC–MS analysis confirmed an approximately 50:50 mixture of 2- and 3-hydroxynonanal. Dilutions of this mixture were made for use as the internal standard and used without further purification.

Sample preparation

Several types of biological samples have been analyzed using this method, namely cultured cells, precipitated proteins, tissues, whole blood, plasma and serum. For the analysis of cultured cells, the media were poured off and the cells collected by scraping into the cold saline. The cells were pelleted by centrifugation, the supernatant discarded and the cells lysed in 2 ml of deionized water. Tissue samples, ranging from 50 to 1000 mg wet weight, were homogenized directly in the oximation reagent described below. Aliquots (0.1–1.0 ml) of whole blood, serum or plasma were analyzed directly under the reaction conditions described below. In each case, an appropriate amount of HNA in methanol was added as the internal standard immediately prior to the oximation reaction.

The oximation was initiated by the addition of 4 ml of 0.3 *M* hydroxylamine hydrochloride in 0.1 *M* acetate buffer, pH 5 with 50 μM butylated hydroxytoluene (BHT) and 1 m*M* diethylenetriaminepentaacetic acid (DETAPAC). The samples were thoroughly mixed with gentle rocking and allowed to react for 1 h at 70°C. The lipid aldehyde oximes were recovered by octadecylsilyl solid-phase extraction. The columns were preconditioned with 3 ml of methylene chloride, 5 ml of methanol and 10 ml of water. The sample was applied to the column and the column washed with 5 ml of water and 2 ml of methylene chloride. The columns were eluted with 5 ml of methanol which was transferred to a 5-ml reaction vial and evaporated to dryness. The residue was re-

constituted in 50 μl of MTBSTFA and 200 μl of DMF and reacted at 70°C for 1 h. A 250-μl volume of saturated NaCl was added and the mixture extracted with two 600-μl aliquots of light petroleum (b.p. 30–65°C). The extracts were combined in a 1-ml reaction vial, evaporated to dryness and reconstituted in 50 μl of isooctane for analysis.

Gas chromatography–mass spectrometry

A double-focusing, reverse-geometry mass spectrometer (Model 8230, Finnigan-MAT, San Jose, CA, USA) was used. The instrument was equipped with a Varian 3700 gas chromatograph with a 30 m × 0.32 mm capillary column with a 0.25 μm film thickness bonded phase polydimethylsiloxane stationary phase (SE-30 Econocap, Alltech Assoc., Deerfield, IL, USA). Injections were made on-column and the gas chromatograph programmed from 100 to 290°C at 15°C/min. The mass spectrometer was operated with 70 eV electron ionization in the SIM mode at a mass resolution of 1500 monitoring m/z 342.2 (4HNE, $[M-57]^+$), m/z 344.2 (HNA, $[M-57]^+$) and using 342.9893 of the reference compound perfluorokerosene as a lock mass. Under these conditions the 4HNE and HNA eluted at approximately 8 min.

Quantitation was determined by integrating the appropriate chromatographic peak areas for 4HNE and HNA. The area ratio of 4HNE/HNA was converted to picomoles of 4HNE using a calibration curve determined from standard amounts of the 4HNE-MOX and measured in the same experiment.

RESULTS AND DISCUSSION

The electron ionization mass spectra of the oxime-*tert.*-butyldimethylsilyl derivatives of 4HNE and HNA are shown in Fig. 2. As can be seen, an ion of high relative intensity is observed for each compound which correponds to the loss of C_4H_9 from the respective molecular ions. This fragmentation pathway is ideal for SIM because these ions contain a large portion of the molecular structure, which promotes specificity, and

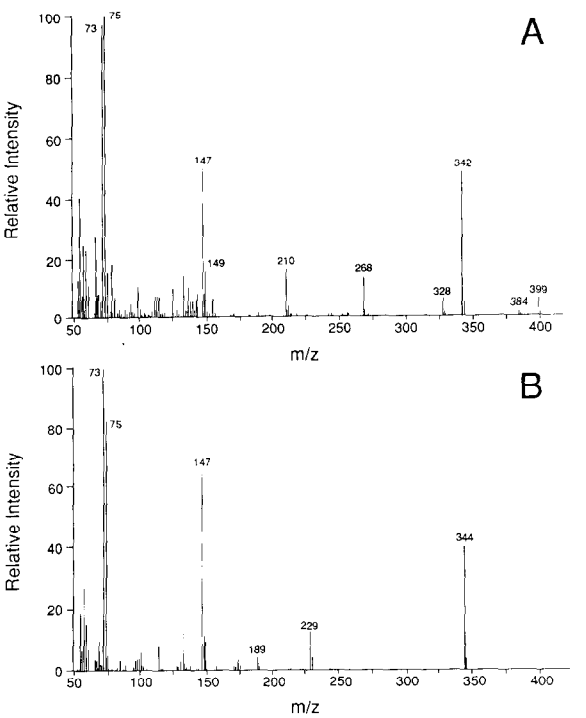

Fig. 2. Electron ionization mass spectra of the oxime-bis-*tert.*-butyldimethylsilyl derivatives of (A) 4-hydroxy-2-nonenal (4HNE) and (B) 3-hydroxynonanal (HNA).

because these ions carry a large portion of the total ion current, which promotes sensitivity. It is also useful that the ions of interest are closely matched in m/z particularly when using a magnetic sector instrument on which changes in the accelerating voltage are made to switch between masses since large mass differences can result in a mass discrimination during this switching. These compounds also have similar chromatographic characteristics eluting within a 20-s time window.

A typical chromatogram is shown in Fig. 3 for the determination of 4HNE in cultured cells. In this sample 45 pmol of 4HNE were detected in approximately $1.5 \cdot 10^6$ cells. An excellent signal-to-noise ratio was seen with few additional chromatographic peaks in the time window in which the analyte and internal standard eluted. Fig. 4 shows a typical chromatogram for the analysis of a 0.5-ml aliquot of SRM 909 found to contain 40.4 pmol of 4HNE. Again, an excellent signal-to-noise ratio was observed in the chromatogram

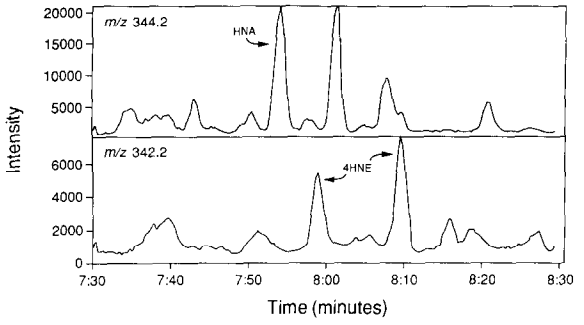

Fig. 3. Selected ion monitoring analysis of 4HNE in culture cells. The analyte, 4HNE, is detected at a retention time of 8:10 min:s in the *m/z* 342.2 ion chromatogram while the internal standard, HNA, is detected at a retention time of 7:54 min:s in the *m/z* 344.2 ion chromatogram. The doublets which are observed are due tot the formation of *syn*- and *anti*-isomers in the oximation reaction.

with few additional peaks. A limit of detection of approximately 1 pmol of 4HNE was observed using this method.

In these chromatograms, the typical appearance of oxime derivatives is seen. The nucleophilic substitution reaction results in the formation of *syn*- and *anti*-isomers which are generally easily resolved by capillary GC. For 4HNE, the doublet of peaks is seen in Fig. 3 at retention times of approximately 7:58 and 8:10 min:s and are cleanly resolved. Quantitative analysis can use the chromatographic peaks of either of the isomers.

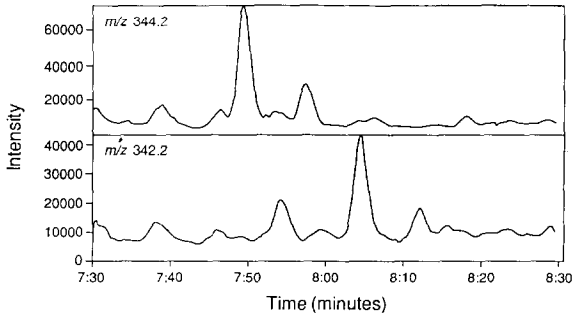

Fig. 4. Selected ion monitoring analysis of 4HNE in National Institute of Standards and Technology Standard Reference Material (NIST SRM) 909. The analyte, 4HNE, is detected at a retention time of 8:05 min:s in the *m/z* 342.2 ion chromatogram while the internal standard, HNA, is detected at a retention time of 7:50 min:s in the *m/z* 344.2 ion chromatogram. The doublets which are observed are due to the formation of *syn*- and *anti*-isomers in the oximation reaction.

For HNA, the doublet of peaks is observed at retention times of 7:50 and 7:54 min:s. In the *m/z* 344 ion chromatogram a second doublet of peaks is observed at retention times of 8:00 and 8:04 min:s. These peaks are due to the formation of 2-hydroxynonanal in the synthetic procedure. This set of peaks would be equally appropriate for use as an internal standard since constant amounts are added along with the constant amounts of 3-hydroxynonanal. However, at times the efficiency of the silylation reaction has been observed to be variable for this compound, possibly due to steric hindrance because of the proximity of the oxime hydroxyl and the 2-hydroxyl in this compound.

The efficiency of the oximation reaction for Schiff bases was investigated using the metoxime derivative of 4HNE as a model compound. The reaction sequence for both the Schiff base-bound 4HNE and the 4HNE-MOX is shown in Fig. 1, reactions b and c. In these experiments, amounts of 4HNE-MOX were reacted with the oxime under a variety of conditions and the product mixture evaluated by GC following conversion to the trimethylsilyl ether. The results are summarized in Table I. As can be seen, reaction at 70°C for 1 h affords a rapid, high-yield conversion of the methoxime to the oxime derivative. High conversion is also achieved by reaction overnight at room temperature, but the efficacy of this reaction at room temperature for 1 h is considerably

TABLE I

OXIMATION REACTION EFFICIENCY

A 10-nmol amount of 4HNE-MOX was reacted with the oximation reagent described in the Experimental section under various conditions. The reaction mixture was extracted, the extract converted to the trimethylsilyl derivative and analyzed by gas chromatography. Relative peak areas were used to determine the percentage conversion. All determinations were done in duplicate.

Reaction conditions	Percentage conversion
Room temperature, 1 h	7.5
70°C, 1 h	91.1
Room temperature, overnight	89.0

less than quantitative. We, therefore, have chosen to react all samples at 70°C for 1 h.

The 4HNE-MOX derivative is less volatile and more resistant to oxidation than the underivatized parent compound. As a result, we have found that the concentration of standard solutions is more stable using 4HNE-MOX than using 4HNE. The 4HNE-MOX derivative still retains a strong chromophore allowing the accurate determination of the concentration of the standards. Since this compound also serves as a effective model for the Schiff base-bound form of 4HNE, all calibration is done using 4HNE-MOX. We feel that this modeling, in combination with the ability to make stable standard solutions of accurately known concentrations, significantly enhances the accuracy of this trace method. A typical calibration curve, for standard amounts of 4HNE-MOX ranging from 0 to 300 pmol, is described by the equation: amount 4HNE (pmol) = 63.2 × peak-area ratio − 2.35; correlation coefficient = 0.996.

In validating our method, we also investigated autooxidation and the potential for the artifactual formation of 4HNE during the extraction and derivatization process. Two different experiments were utilized and the results are summarized in Table II.

In the first experiment, 2-ml aliquots of a 300 m*M* solution of linoleic acid were analyzed with the oximation reaction under a series of time and temperature conditions, both with and without the addition of 50 μM BHT and 1 m*M* DETAPAC as antioxidants. As can be seen from these data, 1 h at room temperature produced a slight increase in the amount of 4HNE observed in the absence of antioxidants relative to the presence of antioxidants. Similar results are seen for the overnight reaction although some oxidation is apparent. However, 70°C for 1 h produced far greater amounts of 4HNE in the absence of the antioxidants indicating the autooxidation of the linoleic acid and the formulation of 4HNE. These results were confirmed in the second experiment using cultured cells. Again the results indicate that the reaction conditions found to be required to quantitatively convert Schiff base-bound 4HNE to the oxime derivative require the addition of antioxidants to prevent artifactual formation of the analyte.

TABLE II

EXTENT OF AUTOOXIDATION UNDER THE OXIMATION CONDITIONS

Two types of samples were reacted either in the presence of 50 μM BHT and 1 m*M* DETAPAC (designated as "with") or in the absence of any antioxidants (designated as "without"), as specified. The analysis was completed as described in the Experimental section. All determinations were made in duplicate.

Reaction conditions	4HNE detected (pmol)
Sample: 300 nmol linoleic acid per 2 ml	
70°C, 1 h, with	70.3
70°C, 1 h, without	>1,000
Room temperature, 1 h, with	53.5
Room temperature, 1 h, without	57.1
Room temperature, overnight, with	65.4
Room temperature, overnight, without	112.2
Samples: 3 · 10^6 cultured fibroblasts yielding 0.6 mg protein for analysis	
70°C, 1 h, with	37.0
70°C, 1 h, without	185.7
Room temperature, overnight, with	40.6
Room temperature, overnight, without	218.4

Precision and accuracy were then determined under these validated analysis conditions. In these experiments, SRM 909, a standard reference material manufactured by the NIST, was used for the determination of precision and accuracy. It is hoped that the use of this material will facilitate the export of this method to other laboratories and allow inter-laboratory comparisons to be made on a sample which is wideley available.

Accuracy was evaluated in a series of standard addition experiments. Typical results are shown in Fig. 5. These results indicate that the method is accurate since the standard addition line has a slope which is not statistically different from 1, a correlation coefficient of 0.994, and an x-intercept of −83.0 pmol/ml. We have found the average concentration of 4HNE in SRM 909 to be 82.1 pmol/ml of the reconstituted material or

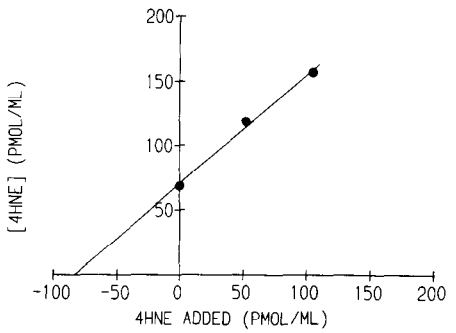

Fig. 5. Standard addition analysis of Standard Reference Material (SRM) 909. The indicated amounts of 4HNE were added to 0.5 ml of SRM 909 and the 4HNE concentration determined as described in the Experimental section. The linear regression line has a slope of 0.84 ± 0.07 and a correlation coefficient of 0.994. All determinations were made in triplicate. Error bars are included but are smaller than the symbols.

97.2 nmol/l/g of the lyophilized powder, reconstituted by NIST procedure A. In the course of these experiments, no differences were observed in the concentration of 4HNE found in three bottles of this material from a single shipment. Although much more extensive study would be needed to further certify the concentration of 4HNE in this material, we feel that SRM 909 does represent an excellent resource for the development of these methods.

Precision was evaluated by replicate analysis of SRM 909 in five sets of three or four replicates over a period of four weeks. These data, summarized in Table III, indicate an average in-run precision of 8.1% with a between-run precision of 13.5%. These results are consistent with this type of analysis.

TABLE III

EVALUATION OF PRECISION

4HNE in Standard Reference Material 909 was measured in replicate analyses as described in the Experimental section. C.V. represents the coefficient of variation.

Replicate	*n*	Concentration of 4HNE (mean ± S.D.) (pmol/ml)	C.V. (%)
1	4	77.3 ± 9.6	12.4
2	3	101.1 ± 7.1	7.0
3	3	86.0 ± 10.6	12.3
4	4	77.8 ± 3.5	4.4
5	3	68.2 ± 2.9	4.3

Average in-run C.V.: 8.1%
Mean-of-means: 82.1 pmol/ml
Between-run standard deviation: 11.1 pmol/ml
Between-run C.V.: 13.5%

CONCLUSION

We have been able to develop a trace analysis method for the lipid aldehyde 4HNE in biological samples in either its free or Schiff base-bound form. The limit of detection is sufficient for the analysis of the sample types encountered in our investigations, including small amounts of whole blood, plasma and serum and small numbers of cultured cells. We have also been able to detect 4HNE in samples of aortic intimal tissue, postnuclear supernatant of rat liver homogenates and homogenates of decapsulated rat testis using this method. The method takes advantage of the formation of an oxime derivative of 4HNE which both liberates the aldehyde from Schiff bases linkages and converts any free aldehyde to the same product. Once formed, the oxime is also easier to handle than the free aldehyde since it is more stable to oxidation and much less volatile. The reduced volatility is important in trace analysis schemes since sample concentration is generally achieved by solvent evaporation. We have also utilized a novel internal standard for this analysis. This internal standard models all the functional group chemistry found in 4HNE and therefore offers some of the advantages of 2,3-d_2-4HNE standard used by others. However, HNA allows accurate and precise quantitation without concern for the stability of the label and therefore would appear to be a better standard. An ideal internal standard would still be isotopically labeled but it is critical that the label be stable in light of the chemistry of the metabolism of 4HNE. The general analysis scheme presented here should be applicable to other aldehydes in either the free or the Schiff base-bound form. Addition of aldehydes are of interest in lipid peroxidation and corresponding methods require only the development of acceptable internal standards.

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