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Urinalysis of 4-hydroxynonenal, a marker of oxidative stress, using stir bar sorptive extraction–thermal desorption–gas chromatography/mass spectrometry

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Abstract

A simple and fast method for the measurement of 4-hydroxynonenal (4HNE), a highly toxic end-product of lipid peroxidation, in urine samples is described. The method combines stir bar sorptive extraction (SBSE) with two derivatization steps, followed by thermal desorption and GC/MS. 4HNE is derivatized in situ with *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine and the oxime is extracted from the aqueous phase with SBSE. The 4HNE-oxime is further acylated by headspace derivatization prior to thermal desorption. Derivatization reactions and extraction were optimized in terms of reagent quantities, temperature and time. The method is linear over a concentration range of 0.5–5 ng mL^{-1} with a correlation coefficient of 0.997. The limit of detection and limit of quantitation are 22 and 75 pg mL^{-1} urine, respectively. The high sensitivity of the method allows the measurement of physiological concentrations of 4HNE in urine samples.
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Keywords: Stir bar sorptive extraction; Gas chromatography–mass spectrometry; 4-Hydroxynonenal; Oxidative stress; Urine samples

1. Introduction

The measurement of aldehydes in biological samples has become increasingly important due to their significance as markers of oxidant injury. Aldehydes are primarily formed in vivo by a free radical-mediated mechanism that is initiated when reactive oxygen species (ROS) such as O_2^- and H_2O_2 interact with cellular membranes [1]. Oxidation of the phospholipid components of these membranes results in the formation of hydroperoxide intermediates that rapidly breakdown to a variety of stable aldehydes, including alkanals, 2-alkenals, 2,4-alkadienals and 4-hydroxyalkenals [2]. One of the most important end-products formed following the oxidation of lipid membranes is the α,β-unsaturated aldehyde, 4-hydroxynonenal (4HNE) [3,4].

4HNE has been studied intensively for many years and found to be highly toxic to mammalian cells [3]. Several adverse effects have been observed in biological tissues when this compound increases above physiological concentrations (i.e. >1 μM). 4HNE has been shown to inhibit DNA, RNA and protein synthesis [5,6], initiate lipid peroxidation [7], rapidly deplete intracellular glutathione [8], and inhibit respiration and glycolysis [9]. These harmful effects have been attributed to the chemical structure of 4HNE, which may explain its high reactivity toward biological substrates [10]. This aldehyde readily reacts with target proteins in biological membranes, forming toxic substances that may lead to the development of chronic diseases. For this reason, 4HNE has been implicated in the pathogenesis of diseases such as atherosclerosis [11], Alzheimer's disease [12], and cancer [13].

Due to the difficulty of measuring ROS directly in vivo, most estimates of oxidative stress rely on the measurement of the breakdown products of lipid peroxidation (e.g. 4HNE). Several analytical methods have been developed for the measurement of 4HNE in biological tissues and fluids, most of which rely on spectrophotometric detection of stable chromophore derivatives. One of the most widely used methods involves the derivatization of 4HNE with 2,4-dinitrophenylhydrazine prior to analysis by HPLC [14,15]. This method offers relatively facile measurement of a number of aldehydes, but its selectivity has been questioned,

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especially when analyzing complex biological matrices where 4HNE occurs at trace levels [16].

The most sensitive analytical methods currently available for the measurement of 4HNE are based on the formation of an oxime derivative using *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBHA) followed by silylation of the hydroxyl group prior to GC/MS analysis [17–19]. The method recently developed by Meagher et al. [20] is sufficiently sensitive to measure physiological concentrations of 4HNE in urine samples. This method was adapted from a procedure that was previously developed for the measurement of 4-hydroxyalkenals in oxidized LDL [21]. All of the previously mentioned methods, however, share a common disadvantage namely that they require tedious sample preparation procedures that involve several extraction and cleanup steps before the derivatives can be analyzed.

New solventless sample-enrichment techniques that allow the direct extraction of solutes from aqueous matrices have recently been introduced such as stir bar sorptive extraction (SBSE) and solid-phase microextraction (SPME) [22,23]. Both techniques combine extraction and concentration of the analytes in a single step, thereby reducing the time required to prepare the samples. A number of reports have already been published for the analysis of carbonyl compounds using SBSE [24] and SPME [25,26]. SBSE has the advantage that higher analytical sensitivities (i.e. >100-fold) can be reached as compared to SPME [22,27], thus favoring this technique for the analysis of trace solutes such as 4HNE. No reports have so far been published on the analysis of hydroxylated-carbonyl compounds using either of these techniques.

One of the limitations in the analysis of compounds containing hydroxyl groups by aqueous SBSE has been the lack of a suitable derivatization method for these analytes. A headspace derivatization technique was recently described by Kawaguchi et al. [28], where BSTFA was added in the thermal desorption tube for in-situ silylation of 17β-estradiol. This method has been based on a concept originally developed by Okeyo and Snow [29], in which SPME fibers were exposed to BSTFA vapors to derivatize steroids that were extracted from urine samples. Shao et al. [30] later extended this technique by replacing BSTFA with acetic acid anhydride for the headspace derivatization of trans-resveratrol in wine. In this contribution, a new SBSE derivatization technique is presented that utilizes acetic acid anhydride catalyzed by pyridine for the headspace derivatization of extracted hydroxy-carbonyl-oximes. The developed method has been applied for the measurement of 4HNE in urine samples.

2. Experimental

2.1. Chemicals and materials

Potassium hydrogen phthalate, butylated hydroxytoluene (BHT), *O*-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine hydrochloride, pyridine and 2,5-dihydroxybenzaldehyde (2,5-DHBA) were purchased from Sigma–Aldrich (Johannesburg, South Africa). Acetic acid anhydride and concentrated sulfuric acid were obtained from Merck (Darmstadt, Germany). 4-Hydroxynonenal (4HNE) was supplied by Cayman Chemical (Kat Medical, Cape Town, South Africa). Methanol was obtained from Riedel-de Haën (Sigma–Aldrich, Johannesburg, South Africa). Five mL glass screw-cap vials (34 mm × 20 mm) and 2 mL glass headspace vials were prepared by E. Ward (University of Stellenbosch). The 5 mL vials were modified from 15 mL screw cap vials obtained from Supelco (Sigma–Aldrich, Johannesburg, South Africa). The 2 mL headspace vials were adapted from 2 mL autosampler vials purchased from Agilent Technologies (Chemetrix, Johannesburg, South Africa). A 10 position magnetic stirrer combined with a convection oven was designed and built by J. Blom and colleagues (Department of Mechanical Engineering, University of Stellenbosch). Twister stir bars (10 mm × 0.5 mm × 0.5 mm d_f polydimethylsiloxane (PDMS)) were purchased from Gerstel GmbH (Müllheim a/d Ruhr, Germany). The stir bars were pre-conditioned by sonication in a 1:1 mixture of dichloromethane:methanol for 5 min after which they were heated at 280 °C for 15 min under a nitrogen flow of 50 mL min^{-1}. The sonicator (Branson 3510) and sterile 50 mL centrifuge tubes were from LASEC (Cape Town, South Africa).

2.2. Solutions

Concentrated solutions of 4HNE (0.1 mg mL^{-1}) and of the internal standard 2,5-DHBA (1 mg mL^{-1}) were prepared in methanol. The solutions were stored at −80 °C and dilutions were made weekly. Standard working solutions were prepared in methanol at concentrations of 1 and 10 μg mL^{-1} for both compounds. Each solution contained 100 μM BHT and was stored at −20 °C. PFBHA was prepared in pyridine at a concentration of 10 mg mL^{-1}. A fresh solution was prepared weekly that was stored at −20 °C.

2.3. Urine samples

Spot urine samples were obtained from 10 volunteers (seven males and three females) that were recruited from students and staff at the University of Stellenbosch. The samples were collected between 9:00 and 12:00 h each day using 50 mL sterile centrifuge tubes that were immediately placed on dry ice before being stored at −80 °C. The volunteers were asked to fill out a short questionnaire to obtain information about their general health and to record their demographic data. Some of the questions that were asked included whether they smoked; were using anti-oxidants; or suffered from any chronic diseases such as asthma or diabetes. The creatinine content in each urine sample was determined by a modification of the Jaffé method [31,32] and the specific gravity (SG) measurements were estimated using an Abbe refractometer (Atago, USA).

The measured 4HNE concentrations were normalized using the corresponding creatinine levels in each sample, and the adjusted concentrations were compared with normalized values that were calculated using a modification of the method of Vij and Howell [33]. These authors recently introduced a new equation for normalizing the excretion of xenobiotic biomarkers in spot urine samples [34]. Using a slight modification of

their method the SG-normalized concentrations of 4HNE were calculated as follows:

$$[4\text{HNE}]_{sz} = \frac{[4\text{HNE}]}{(1.020 - 1/\text{SG} - 1)}(Z) \quad (1)$$

where $[4\text{HNE}]_{sz}$ was the SG- and *Z*-normalized urine samples. The value 1.020 was defined as the mean SG of normal human urine and the respective *Z*-values were calculated by plotting the log[4HNE] values against the log(SG-1) of the individual samples as shown below:

$$Z = \frac{\Delta \log[4\text{HNE}]}{\Delta \log(\text{SG} - 1)} \quad (2)$$

where *Z* corresponded to the slope of the graph. The SG-normalized values of 4HNE were adjusted using the corresponding creatinine concentration in each sample Eq. (3):

$$[4\text{HNE} - \text{Creat}]_{sz} = \frac{[4\text{HNE}]_{sz}}{[\text{Creat}]}(113.12) \quad (3)$$

where $[4\text{HNE-Creat}]_{sz}$ was the creatinine, SG- and Z-normalized urine samples. The symbol [Creat] corresponded to the creatinine concentration (mmol L^{-1}) in the sample and the value 113.12 is the molecular weight of creatinine.

2.4. Sample preparation, in-situ derivatization and SBSE procedure

Urine samples were allowed to thaw overnight at 4 °C after which 1 mL aliquots were transferred to 5 mL glass screw-cap vials containing 1 mL 1 M potassium hydrogen phthalate and 0.52 mL 1 μM BHT in methanol. The BHT was added to the samples to prevent artificial formation of 4HNE during the sample work-up procedure. Each sample was spiked with 1 ng of the internal standard 2,5-DHBA, corresponding to a final concentration of 0.18 ng mL^{-1} in the sample mixture. The pentafluorobenzyl-oxime derivatives of 4HNE and of the I.S. were synthesized by adding 150 μL of 40 mM PFBHA in pyridine. The vials were capped, vortexed gently for 10 s and placed in a sonicator bath for 15 min. Following sonication, 3 mL deionized water and 20 μL concentrated sulfuric acid was added before placing a conditioned stir bar in the sample solution. The pH of the solution was ca. 5.5 prior to the extraction of the oxime derivatives. The samples were stirred at 1100 rpm for 50 min using a home-built magnetic stirrer/oven that was heated up to 42 °C.

2.5. Headspace derivatization

Following SBSE extraction of the pentafluorobenzyl (PFB)-oxime derivatives, the stir bars were removed from the sample vials, washed with distilled water and dried with tissue paper. The stir bars were transferred to cups attached inside 2 mL headspace vials (Fig. 1) and 20 μL acetic acid anhydride and 20 μL pyridine were added. The vials were incubated at 70 °C for 20 min to form the acetate derivatives of the extracted compounds.

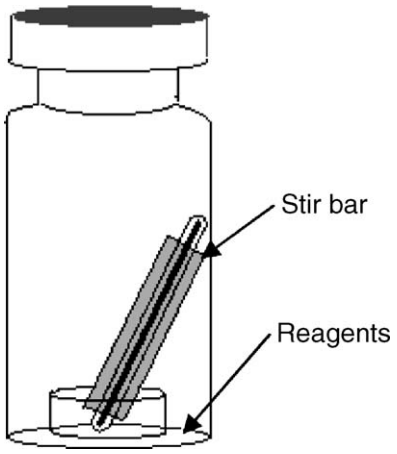

Fig. 1. Modified autosampler vial (2 mL) used for headspace acylation of the SBSE extracted 4HNE-oxime derivatives.

2.6. Thermal desorption–gas chromatography–mass spectrometry

The stir bars were removed from the headspace vials, washed and dried, and placed in glass desorption tubes of a TDS A2 autosampler (Gerstel). Thermal desorption was accomplished in the solvent-venting mode using helium at a flow rate of 60 mL min^{-1}. The temperature of the TDS 2 was increased from 50 to 270 °C (held for 10 min) at a rate of 60 °C min^{-1}. The split-valve was closed after 2 min. The desorbed compounds were transferred to a programmable temperature vaporization (PTV) inlet (CIS 4, Gerstel), and were cryofocussed at 5 °C using liquid nitrogen. The TDS transfer line was held at 280 °C. Sample injection onto the capillary column was accomplished by programming the PTV from 5 to 280 °C (held for 5 min) at a rate of 12 °C s^{-1}.

GC/MS analyses were carried out on a Agilent 6890 gas chromatograph that was interfaced with a 5973N mass selective detector (Agilent Technologies, Little Falls, DE, USA). The derivatives were separated on a HP-5MS fused silica capillary column (30 m × 0.25 mm × 0.25 μm, Agilent) using helium as carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature was programmed from 70 °C (held for 1 min) to 145 °C at 20 °C min^{-1}, and from 145 to 300 °C at 8 °C min^{-1}. The solvent delay was 9.5 min and the transfer line temperature was maintained at 280 °C. Mass spectra of the derivatized compounds were first recorded in full scan, whereas quantification was performed in the selected ion monitoring (SIM) mode. The electron ionization voltage was 70 V. For SIM, three ions characteristic for the analytes were monitored at 100 m s^{-1} each, namely *m/z* 322, 351 and 393 for 4HNE and *m/z* 316, 333 and 375 for 2,5-DHBA (internal standard). The underlined values are the ions used for quantification.

3. Results and discussion

3.1. SBSE–HD–TD–GC/MS determination of 4HNE

The presented method is based on the analysis of 4HNE in urine samples using SBSE–headspace derivatization–thermal

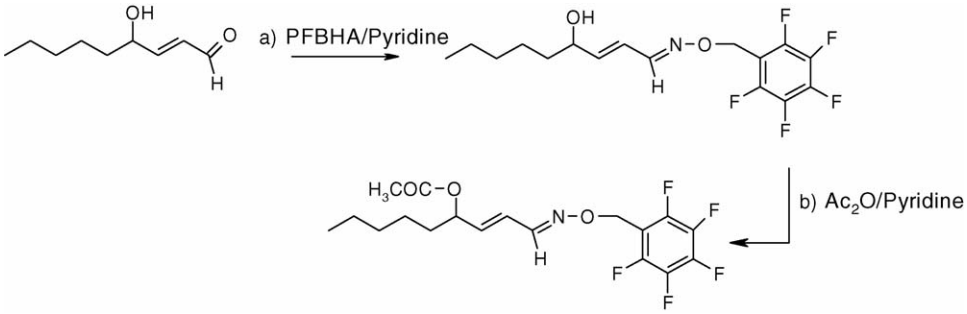

Fig. 2. Reaction scheme of the two-step derivatization method for 4HNE using: (a) PFBHA in the aqueous sample matrix and (b) acetic acid anhydride in the headspace to form the 4HNE-oxime-acetate derivatives. Conditions are given in Section 2.

desorption–GC/MS (SBSE–HD–TD–GC/MS). The 4HNE-oxime derivatives were prepared by a reaction with PFBHA in the aqueous sample matrix, followed by extraction of the derivatives using SBSE. Thereafter, the acetate derivatives of the extracted compounds were formed in the polydimethylsiloxane phase by exposing the stir bars to acetic acid anhydride vapors at 70 °C. Pyridine was used as a catalyst in both reaction steps as shown in Fig. 2. The PFB-oxime-acetate derivatives were thermally desorbed and analyzed on-line by GC/MS.

Initial experiments were performed with pure water samples containing 4HNE and the internal standard at a concentration of 0.5 μg mL^{-1} to determine the retention times and mass spectra of the respective PFB-oxime-acetate derivatives. Electron impact mass spectra of 4HNE and 2,5-DHBA (Fig. 3) show the molecular ion of 4HNE (*m/z* 393), and that of the I.S. (*m/z* 417). The spectra of both compounds are characterized by the initial loss of [CH_2=C=O], resulting in *m/z* 351 for 4HNE and *m/z* 375 for 2,5-DHBA. The loss of a second [CH_2=C=O] group from 2,5-DHBA results in ion *m/z* 333, that is the base peak of this derivative. The peak of highest intensity for 4HNE is ion *m/z* 181, which is characteristic for PFB-derivatized compounds analyzed by electron impact mass spectrometry. Using this ion for SIM analyses of aldehyde-oxime derivatives is believed to lack specificity because many other compounds, including ketones, are present in biological fluids [16]. Ions were selected that are highly characteristic for 4HNE to ensure adequate separation and specific detection of this aldehyde in complex matrices such as urine. Fig. 4 shows the SIM chromatograms of the derivatized compounds in water (A) and in a urine sample (B). For the water sample 1 ng mL^{-1} of 4HNE and I.S. were added while only 1 ng mL^{-1} I.S. was added to the urine sample. The figures indicate that the selected ions are highly specific and that the urine matrix does not disturb the quantification. Note that 4HNE consists of a *syn* and *anti*-isomer peak while a single peak is observed for the I.S. [18]. Quantification of 4HNE was performed using the second isomer peak, as the first peak was not always separated when different urine samples were analyzed.

The sorption of compounds by the stir bar depends largely on the partition coefficient of the solutes between the polydimethylsiloxane layer covering the stir bar and the aqueous phase. By using the octanol-water distribution coefficient ($K_{o/w}$), which is comparable to the partition coefficient ($K_{PDMS/w}$), it is possible to predict the enrichment factor for a given compound from an aqueous solution [35]. The log $K_{o/w}$ values of 4HNE (non-oximated and oximated) were calculated using the Log P predictor which is available from Interactive Analysis Inc. (Bedford, MA, USA) and are 1.35 and 2.48, respectively. The enrichment factors at equilibrium estimated with the Twister-Calculator available from RIC (Kortrijk, Belgium) are 8.6 and 56%, respectively for the 0.5 mm PDMS layer. Enrichment of 4HNE from aqueous matrices is thus significantly enhanced when the 4HNE-oxime derivative is formed prior to performing SBSE.

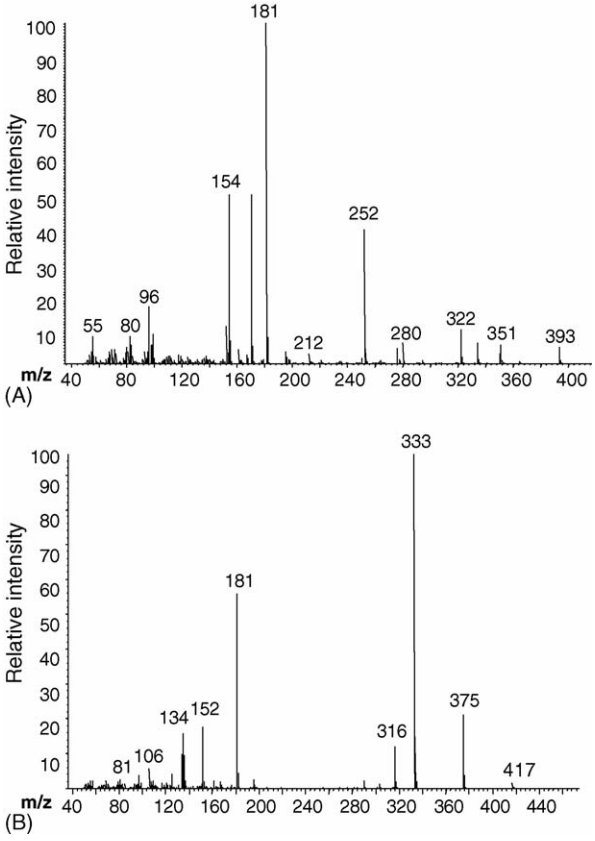

Fig. 3. Mass spectra of the PFB-oxime-acetate derivatives of: (A) 4HNE and (B) 2,5-DHBA (I.S.). The selected ions used to construct the chromatograms in Fig. 4 were *m/z* 322, 351 and 393 for 4HNE and *m/z* 316, 333 and 375 for 2,5-DHBA.

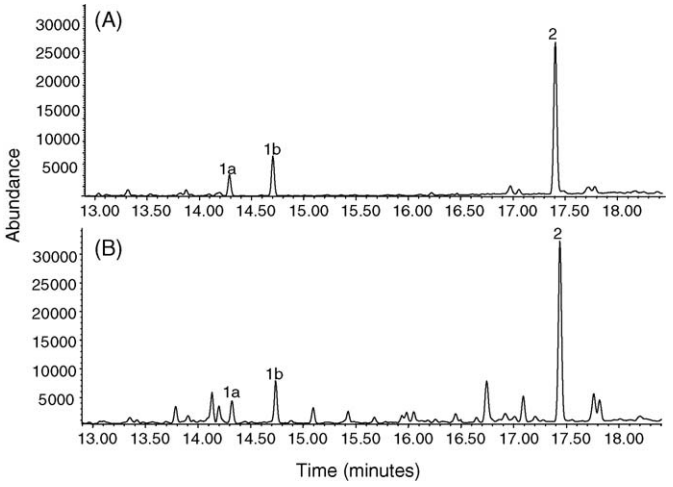

Fig. 4. SIM chromatograms by SBSE–HD–TD–GC/MS of the PFB-oxime-acetate derivatives of: (1a,1b) the *syn*- and *anti*-isomers of 4HNE and (2) 2,5-DHBA (I.S.). The upper trace (A) corresponds to the analysis of a 1 mL water sample containing 1 ng of 4HNE and 1 ng of the I.S.; and the lower trace (B) corresponds to the analysis of a 1 mL urine sample spiked with 1 ng of the I.S.. Extraction, derivatization and chromatographic conditions are described in Section 2.

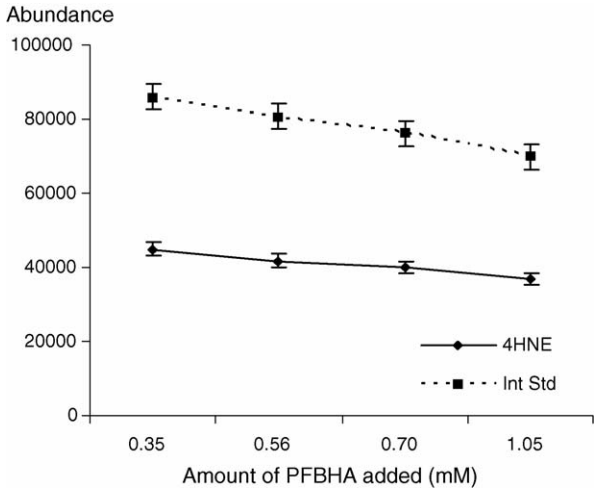

Fig. 5. Amount of PFBHA used to form the oxime-derivatives of 4HNE and the I.S.. A 1 mL urine sample spiked with 1 ng of both compounds was analyzed by SBSE–HD–TD–GC/MS. Conditions are given in Section 2.

3.2. Method optimization

The different steps of the derivatization reactions and SBSE extraction were optimized in order to obtain the highest yields and this combined with good reproducibility. All experiments were conducted in urine samples to which an extra 1 ng mL^{-1} of 4HNE and 1 ng mL^{-1} of the I.S. standard were spiked. The samples were analyzed by SBSE–HD–TD–GC/MS using the conditions described in the experimental section.

Firstly, the PFBHA reaction was optimized and compared with two PFBHA derivatization methods that were recently published [20,26]. Different amounts of the reagent were added to the sample matrix, namely 0.35, 0.56, 0.70 and 1.05 mM PFBHA and the reaction was allowed to proceed as described in the experimental section. SBSE was performed at 42 °C during 50 min. The results are shown in Fig. 5.

The use of 0.35 mM PFBHA resulted in higher yields of the PFB-oxime derivatives as compared to using 1.05 mM PFBHA. Using lower amounts of the reagent seemed advantageous because fewer by-products of the PFBHA reaction were extracted by the stir bar. However, it was noticed that the reproducibility of the method was influenced by the amount of reagent used. Reproducibility ($n = 3$) was improved at higher concentrations of PFBHA (3% for 1.05 mM PFBHA versus 8% for 0.35 mM PFBHA). Therefore, 1.05 mM PFBHA was chosen for the derivatization of 4HNE. Using these conditions, the effect of time on the reaction yield of the PFB-oxime derivatives was assessed. Samples were sonicated for 15 min, placed in the dark for 30 min and left in the dark for 2 h. Comparable yields of the 4HNE-oxime derivatives were obtained using the three methods.

The time required for the PFB-oxime derivatives to reach extraction equilibrium was studied by varying the extraction time from 30 to 120 min. The peak areas for 4HNE and the I.S. reached an optimum at ca. 50–60 min. While the peak area of the I.S. slightly declined in the time interval 60–120 min, a sharp rise was observed in the extracted amounts of 4HNE between 90 and 120 min, indicating that 4HNE could be artificially formed in the urine sample. This was further investigated by varying the extraction temperature from 40 to 70 °C. This experiment was also performed using pure water samples to control for the surplus formation of 4HNE in urine samples. The extraction of the I.S. followed the same decreasing trend in both sample matrices, but an increased amount of 4HNE was extracted from the urine sample in the temperature interval 50–70 °C. Therefore, it was concluded that higher extraction temperatures, as well as prolonged extraction times, may result in artificial formation of 4HNE. For this reason, an extraction temperature of 42 °C and a stirring time of 50 min were chosen to prevent the artificial increase of 4HNE during the extraction process.

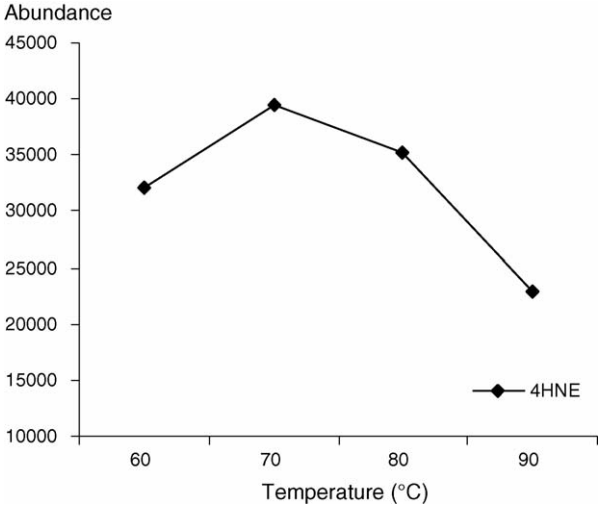

Fig. 6. Headspace derivatization temperature. A 1 mL urine sample containing 1 ng of 4HNE was analyzed by SBSE–HD–TD–GC/MS. Conditions are given in Section 2.

Secondly, parameters that affect the headspace acylation of the extracted PFB-oxime derivatives namely temperature and time were evaluated. The stir bars were exposed to acetic acid anhydride vapors at various temperatures (i.e. 60–90 °C) to determine the efficiency of the derivatization reaction at 20 min exposure time. Fig. 6 illustrates that 70 °C resulted in the highest conversion of the oxime derivatives to the corresponding oxime-acetates. The optimum time required to form the acetate derivatives of the extracted compounds was investigated by exposing the stir bars to the acetic acid anhydride vapors for various lengths of time, i.e. 10–60 min. An optimal yield was obtained at 20 min.

3.3. Figures of merit

The optimized SBSE–HD–TD–GC/MS procedure was validated with regard to linearity, detection and quantitation limit, precision and relative (versus I.S.) recovery. Calibration curves were obtained by spiking 1 mL urine samples with increasing amounts of 4HNE, namely 0.5, 1, 3 and 5 ng mL^{-1}. A fixed amount of the internal standard was added (i.e. 1 ng mL^{-1}). The slope of the curve was obtained by plotting the peak area ratios of 4HNE corrected for the I.S. against the concentration of the analytes. The method was linear over the chosen concentration range with a correlation coefficient (r^2) of 0.997. The detection and quantitation limits were calculated using the peak areas of 4HNE in urine samples of known concentration. The LOD corresponded to 22 pg mL^{-1} urine at a signal-to-noise level (S/N) of 3, whereas the LOQ was estimated at 75 pg mL^{-1} urine (S/N = 10). The precision of the SBSE method was determined by calculating the relative standard deviation (R.S.D.%) of five replicate analyses that were made on 3 different days in urine samples containing 1 ng mL^{-1} 4HNE. The intra-day repeatability was 3.3% and the inter-day repeatability was 2.8, 5.6 and 7.2%, respectively. The recovery was calculated by adding 4HNE to urine samples at two different concentration levels namely 0.8 and 2 ng mL^{-1}. The results were obtained by subtracting the peak areas obtained for the spiked urine samples from those obtained for the non-spiked samples. The calculated recoveries were 95 and 104% for each of the spiked levels. A summary of the figures of merit is shown in Table 1.

Table 1
Figures of merit obtained for 4HNE analyzed by SBSE–HD–TD–GC/MS

Parameter	Result
LOD (pg mL^{-1})[a]	22.5
LOQ (pg mL^{-1})[b]	75
Correlation coefficient (r^2)	0.997
Recovery (%)	
0.8 ng mL^{-1}	95
2 ng mL^{-1}	104
Intra-day repeatability (RSD%)	3.3
Inter-day repeatability (RSD%)	7.2

[a] Limit of detection.
[b] Limit of quantitation.

3.4. Measurement of 4HNE in urine samples

Urine samples obtained from 10 volunteers were analyzed by the developed SBSE–HD–TD–GC/MS method. The measured 4HNE concentrations in most of the samples were below 1 ng mg^{-1} creatinine, but two samples showed highly elevated levels of 4HNE (Table 2). One of the volunteers suffered from asthma, whereas the other had smoked heavily for a number of years. Both conditions have previously been associated with increased levels of oxidative stress [36,37]. However, it is not known whether these specific concentrations could be attributed to asthma or smoking alone. More in-depth and broader investigations are required. No apparent agreement could be made between the use of antioxidants and the reported 4HNE concentrations, but no information was given regarding the type of antioxidants used or the frequency of their use. The mean ± S.D.

Table 2
Summary of data obtained for the analysis of urine samples by SBSE–HD–TD–GC/MS

No.	Age	Gender	4HNE (ng mL^{-1})[a]	4HNE-Creat[b]	4HNE-SG-Creat[c]	Antioxidants
1	19	M	0.31	0.20	0.31	+
2	20	F	0.39	0.29	0.41[d]	–
3	19	M	0.35	1.08	0.46	+
4	21	M	1.41	1.07	1.37[e]	+
5	19	M	0.62	0.35	0.52	–
6	19	M	0.25	0.14	0.25	+
7	23	F	0.32	0.19	0.30	–
8	28	M	0.36	0.52	0.33	+
9	39	M	2.48	1.73	2.45[f]	+
10	20	F	0.68	0.42	0.54	–

Response to the use of antioxidants is included.
[a] Actual 4HNE concentrations measured by SBSE–HD–TD–GC/MS.
[b] 4HNE normalized for the creatinine concentration in each sample (ng mg^{-1} creatinine).
[c] 4HNE normalized for urine specific gravity using Eqs. (1) and (2) in Section 2.3. These values were then corrected for the creatinine content in the samples using Eq. (3) (ng mg^{-1} creatinine).
[d] Smoked occasionally.
[e] Person suffering from asthma.
[f] Daily smoker.

for the seemingly healthy volunteers (i.e. those that had 4HNE levels below 1 ng) were 0.39 ± 0.11 ng mg^{-1} creatinine. These concentrations are in agreement with previously published results obtained for healthy controls [20].

This study describes the analysis of 4HNE in random-collected spot urine samples. The measured amounts were normalized to reflect the overall 24 h excretion of 4HNE and to control for possible urine concentration effects. A number of normalization techniques were considered. The most commonly used method involves adjustment of the measured concentration against the creatinine content in the sample. However, this method has been criticized because creatinine excretion has been shown to vary considerably over short intervals [38]. The modified specific gravity (SG) normalization technique of Vij and Howell [33] appeared to be the most promising method. This technique incorporates SG and creatinine, and has been shown to result in the lowest between-subject variability for xenobiotic biomarkers measured in spot urine samples of smokers [34]. However, the values calculated by this method did not always compare well with 4HNE values obtained by the creatinine normalization technique (results not shown). Therefore, we investigated using a modification of the equation introduced by Vij and Howell (Section 2.3), which gave much improved results. It was also observed that the values calculated by this modified equation were highly correlated with the actual measured concentrations of 4HNE, as compared to values that were obtained by the creatinine normalization technique (see Table 2). Nevertheless, the application of this equation for the normalization of 4HNE in spot urine samples should be validated.

4. Conclusion

4HNE is one of the most cytotoxic end-products of lipid peroxidation. Reliable, non-invasive measurements of this aldehyde are required to further elucidate the role of free radicals in the pathophysiology of human diseases. In this investigation, a highly sensitive analytical method was developed for the trace measurement of 4HNE in urine samples. The method combines a solventless sample enrichment technique, namely SBSE, with two derivatization steps, followed by thermal desorption and GC/MS. Detection limits of 22 pg mL^{-1} urine could be achieved, which is much lower than the physiological quantities of 4HNE excreted daily. Furthermore, the measurement of 4HNE in random-collected spot urine samples has several advantages, such as convenience, preservation of sample integrity and greater compliance among participants. These advantages and the simplicity of the analytical technique highlight the potential that this method could be applied to larger clinical trials.

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