Food Anal. Methods (2014) 7:836–843
DOI 10.1007/s12161-013-9689-2

Development of a Sensitive and Accurate Stable Isotope Dilution Assay for the Simultaneous Determination of Free 4-Hydroxy-2-(*E*)-Nonenal and 4-Hydroxy-2-(*E*)-Hexenal in Various Food Matrices by Gas Chromatography–Mass Spectrometry

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Received: 3 June 2013 / Accepted: 19 July 2013 / Published online: 13 August 2013

Abstract An analytical method suitable for the determination of 4-hydroxy-2-(*E*)-nonenal (HNE) and 4-hydroxy-2-(*E*)-hexenal (HHE) in various food matrices was developed and validated. The method involves the use of deuterated HNE and HHE as internal standards, extraction of the analytes from the matrices followed by derivatization and detection with gas chromatography–mass spectrometry. Four different food matrices were chosen as model systems including vegetable oils, unprocessed meat, fried potato crisps, and infant formula and three different extraction techniques suitable for the different matrices were applied including the Quick Easy Cheap Effective Rugged Safe method. The simplicity of the extraction techniques allows the method to be applied for routine analysis of a large amount of samples. The results verify the accuracy and reproducibility of the analytical technique and its ability to provide reliable quantification of both analytes at concentrations as low as 12.8 ng g^{-1} in meat samples. Furthermore, a short overview of the levels of HNE and HHE in several products available in the Belgian market is presented.

Keywords Lipid oxidation · 4-Hydroxy-2-(*E*)-nonenal · Hydroxy-2-(*E*)-hexenal · QuEchERS

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Introduction

One of the main causes of chemical deterioration of foods, especially those containing polyunsaturated fatty acids (PUFA), is lipid oxidation (Belitz et al. 2009). Oxidation of unsaturated fatty acids leads to the formation of odorless and tasteless hydroperoxides, which further decomposed to flavorful secondary oxidation products mainly aldehydes. Hydroxylated unsaturated aldehydes such as 4-hydroxy-2-(*E*)-nonenal (HNE) and 4-hydroxy-2-(*E*)-hexenal (HHE) have attracted the attention due to their toxicity to humans which is attributed to their high reactivity with proteins and DNA, consequently leading to structural damage and alteration of their functionality (Kinter 1995; StAngelo 1996; Esterbauer et al. 1991; Esterbauer 1982; Guillen and Goicoechea 2008a; Voulgaridou et al. 2011; Goicoechea et al. 2008; Goicoechea et al. 2011). HNE is formed during the oxidation of ω-6 PUFA, while HHE is mainly related to the oxidation of ω-3 PUFA (Long and Picklo 2010; Surh et al. 2010; Han and Csallany 2009; Pryor and Porter 1990; Guillen and Uriarte 2012); thus, it can be easily understood that foods rich in PUFA can be a major intake source of HNE and HHE (Surh and Kwon 2005).

Several studies have been published on detection and quantification of unbound 4-hydroxyalkenals from foods and oils. Some involve the extraction and derivatization of 4-hydroxyalkenals followed by analysis with HPLC and UV detection (Seppanen and Csallany 2002; Seppanen and Csallany 2001), HPLC-MS/MS (Zanardi et al. 2002), gas chromatography–mass spectrometry (GC-MS) (Surh and Kwon 2002, 2003), HS-SPME-GC-MS (Guillen et al. 2009; Guillen and Goicoechea 2008b), or GC coupled with electron capture detection (Santaniello et al. 2007). The analytical methods presented in the aforementioned studies, with the

exception of those applying SPME, involve several extraction steps in which loss of a portion of the analyte can be expected, leading to an underestimation of the actual amount present in the sample. Furthermore, in some cases, the extraction techniques applied are time consuming and, when complex food matrices are analyzed, interaction between the analytes and particularly proteins are not taken into account (Han and Csallany 2009, 2012). Among the available techniques in use for determination of aldehydes in foods, GC-MS is the most attractive one. On one hand, it has the advantage of the powerful chromatographic separation provided by gas chromatography, and on the other hand it offers the option of using isotopically labeled HNE and HHE as internal standards (IS). Addition of IS into the samples prior to the analysis potentially minimizes both underestimation of the analytes and variation between the sample replicates. Such techniques have been previously reported in the analysis of HNE and HHE in biological samples (Rees et al. 1995; Vankuijk et al. 1995) and human milk (Michalski et al. 2008) using deuterium labeled HNE and HHE. Recently, LaFond et al. (2011) have published an analytical method for determination of HNE in frying oils that involved the addition of deuterated HNE as an IS prior to the extraction steps. However, there is no available reliable analytical method in the literature for determination of HNE and HHE in more complex proteinous foods such as dairy products, meat, fish, or snacks which also takes into account the interferences of the matrix during the analysis.

In this study, a method for simultaneous determination of HNE and HHE in various food matrices, employing GC-MS and deuterated isotopes as IS, has been developed and validated. It is the first time, to our knowledge, that a method combining HNE and HHE determination in several foods has been reported. Furthermore, a brief overview on the levels of HNE and HHE in several fatty foods available in the Belgian market is provided.

Experimental

Supplies and Reagents

HNE and HHE standards were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA) and both were of 98 % purity according to the certification provided by the supplier. HNE *d11* and HHE *d5* dimethyl acetals were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada). Chemical purity and isotopic enrichment were 90 and 98 %, respectively, for HNE, and 95 and 99 %, respectively, for HHE, according to the certification provided by the supplier. *O*-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA) was supplied by Sigma-Aldrich (Bornem, Belgium) and N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were obtained by Acros Organics (Geel, Belgium). Deionized water (Milli-Q, Millipore Corp.) of 18.0 MΩ cm^{-1} resistivity was used throughout the experiments and all the solvents used were of analytical grade. All the foods used in the experiments were purchased from the local market and were analyzed as soon as they were unpacked. The dry nuts were packed under modified atmosphere. To simulate conditions occurring during frying, 5 g of commercially available ω-3 enriched frying oil (4 % of the total fatty acids) and regular frying oils (marketed as such and were not containing ω-3 fatty acids) were placed in glass test tubes without a cap and were heated in an oven at 170 °C for 5 h.

Extraction Techniques

The foods chosen for development and validation of the analytical method were vegetable oil (palm kernel oil), infant formula, potato crisps, and unprocessed beef meat. Three different extraction procedures for HNE and HHE were applied depending on the food matrix. In order to evaluate the efficiency of the extraction techniques to recover the analytes from the matrices and evaluate any matrix effect, addition curves were prepared. Samples were spiked with 0.36, 0.28, 0.2, 0.12, and 0.04 μg of HNE and HHE and 0.2 μg of HNE *d11* and HHE *d5* was added as IS. HNE and HHE standards were dissolved in methanol, HNE *d11* and HHE *d5* dimethyl acetals were deprotected prior to use by dissolving in 0.5 mL of 20 mM HCl and kept at 4 °C for 1 h, following the instructions of the supplier. A total of 20 μL of the HCl solution containing the deprotected IS was added in the samples.

For oils, 500 mg sample were weighed in a 15-mL polypropylene tube, spiked with the analytes and the internal standards, 0.5 mL of hexane was added, and the mixture was vortexed (VWR, Leuven, Belgium) for 1 min in order to achieve complete incorporation of the analytes and the IS. Two milliliters of water/methanol (60/40) were added and the sample was vortexed for 2 min. The tubes were centrifuged at 2,000×*g* for 2 min (Sigma 4K15, Sartorius, Goettingen, Germany) and 1 mL of the aqueous portion was collected and subjected to derivatization.

For infant formulas, a modified procedure as described by Payá et al. (2007) known as Quick Easy Cheap Effective Rugged Safe (QuEChERS) was applied. A total amount of 500 mg sample was weighed in a 15-mL polypropylene tube, spiked with the analytes and the IS, added with 2 mL of water, and the mixture was vortexed for 1 min. A total of 3 mL of acetonitrile was added followed by vortexing for 1 min. A total of 0.3 g of NaCl and 1 g of $MgSO_4$ were added and the tubes were mixed manually for one more minute. The tubes were centrifuged at 3,600×*g* for 5 min and 2 mL of the supernatant was collected and evaporated under a gentle flow of nitrogen prior to derivatization.

Concerning potato crisps, a total of 500 mg of sample previously ground was weighed in a 15-mL polypropylene tube, spiked with the analytes and the IS. A total of 5 mL of 1.66 M H_2SO_4/methanol (60/40) was added and the mixture was vortexed for 2 min. The tubes were centrifuged at 3,600×*g* for 5 min and the aqueous phase was filtered through a wet 150-mm filter paper. Filtrate (2.5 mL) was collected and subjected to derivatization. The same procedure was followed for the beef samples, with the difference that 1 g of sample was homogenized with an Ultra Turax (Janke and Kunkel, IKA-Werk, Staufeb, Germany) for 1 min at 18,000 rpm after spiking.

External calibration curves were prepared by applying the corresponding extraction technique for each matrix excluding the sample. All the extractions were performed in duplicate. The exact amount of HHE and HNE standards used in the experiments was determined by measuring the absorbance at 220 and 223 nm, respectively (Cary 50 UV–vis, Varian, Sint-Katelijne-Waver, Belgium). The mass extinction coefficient provided by the supplier is 16,000 M^{-1} cm^{-1} for HHE and 13,750 M^{-1} cm^{-1} for HNE.

Derivatization Procedure

The extracts were mixed with 1 mL of methanolic solution of 4 mg mL^{-1} PFBHA reagent in a screw-caped glass tube and the samples were incubated for 1 h at 40 °C. The formed oximes were extracted two times with 2 mL of pentane and dried over sodium sulfate prior to collection in a 25-mL pear-shaped evaporation flask. The solvent was evaporated in a rotary evaporator (Heidolph Instruments GmbH & Co, Schwabach, Germany) at 30 °C and the remaining oximes were quantitatively transferred to a glass vial with 200 μL of pentane. Subsequently, the solvent was evaporated under a gentle stream of nitrogen. For silylation, 20 μL of 10 % TMCS in BSTFA and 80 μL of pyridine were added. The mixture was vortexed for 1 min and 1 μL was injected in the GC-MS.

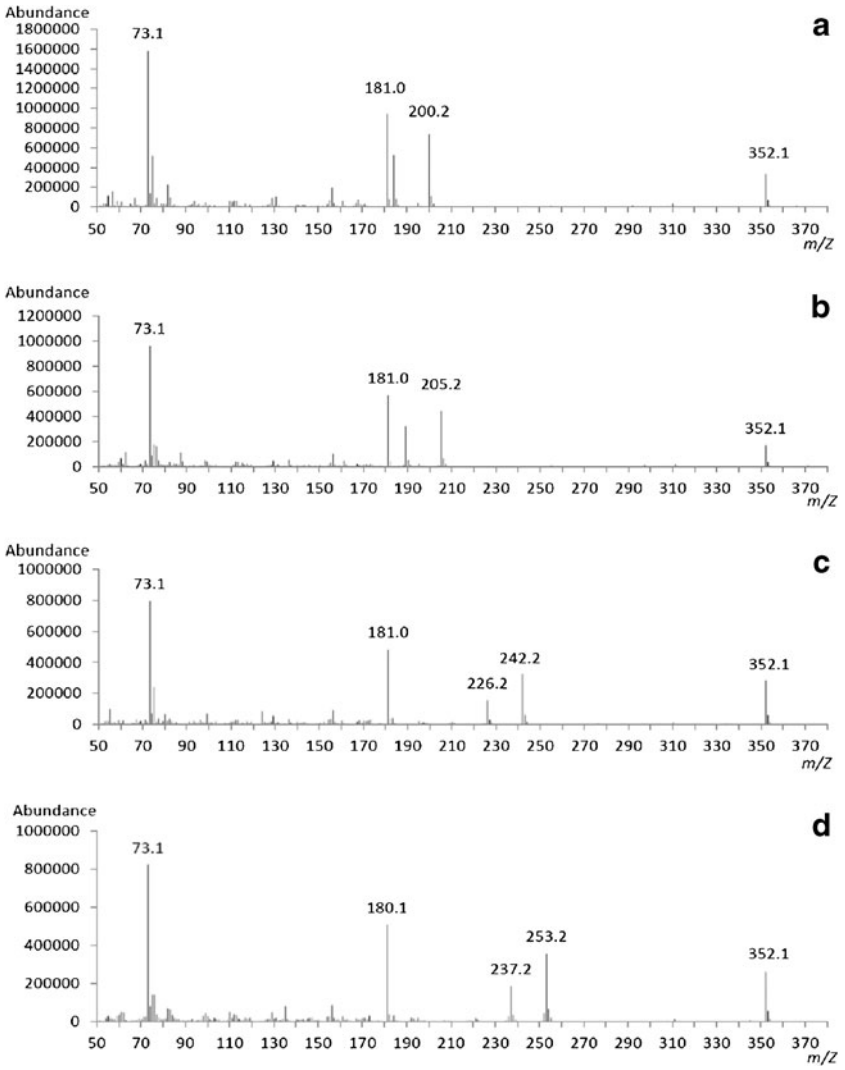

Fig. 1 Mass spectra of **a** HHE; **b** HHE-*d5*; **c** HNE; and **d** HNE-*d11*. The mass spectra were obtained after injection of derivatized standards and deuterated analogues

Instrumental Analysis

Chromatographic analysis was performed in an Agilent 7890A GC equipped with a 5975C Mass Spectrometer (Agilent Technologies, Palo Alto, CA). The derivatized sample (1 μL) was introduced into the injector operating in the splitless mode at 200 °C and the separation was carried out in an Agilent HP-5 MS 30 m, 0.25 mm, 0.1 μm capillary column. The carrier gas was helium at a constant flow of 0.8 mL min^{-1} and the oven temperature was programmed from 50 (held for 1 min) to 150 °C at a rate of 10 °C min^{-1}, from 150 to 200 °C at a rate of 3 °C $min^{-1}$and finally up to 250 °C at a rate of 40 °C min^{-1}. The MSD conditions were the following: capillary direct interface temperature, 250 °C; ionization energy, 70 eV; operating in selective ion mode (SIM); selected ions monitored, *m*/*z* 200, *m*/*z* 205, *m*/*z* 242, *m*/*z* 253, and *m*/*z* 352; scan rate 3.64 cycles/s. In order to obtain the full mass spectra of the HNE, HHE, and their deuterated isotopic oximes, a full scan analysis was performed between *m*/*z* 50 and *m*/*z* 400.

Statistical Analysis

One-way analysis of variance was applied to detect differences between the slopes of the addition curves. All the analysis was carried out with SPSS 18 statistics package (IBM, SPSS, Inc.).

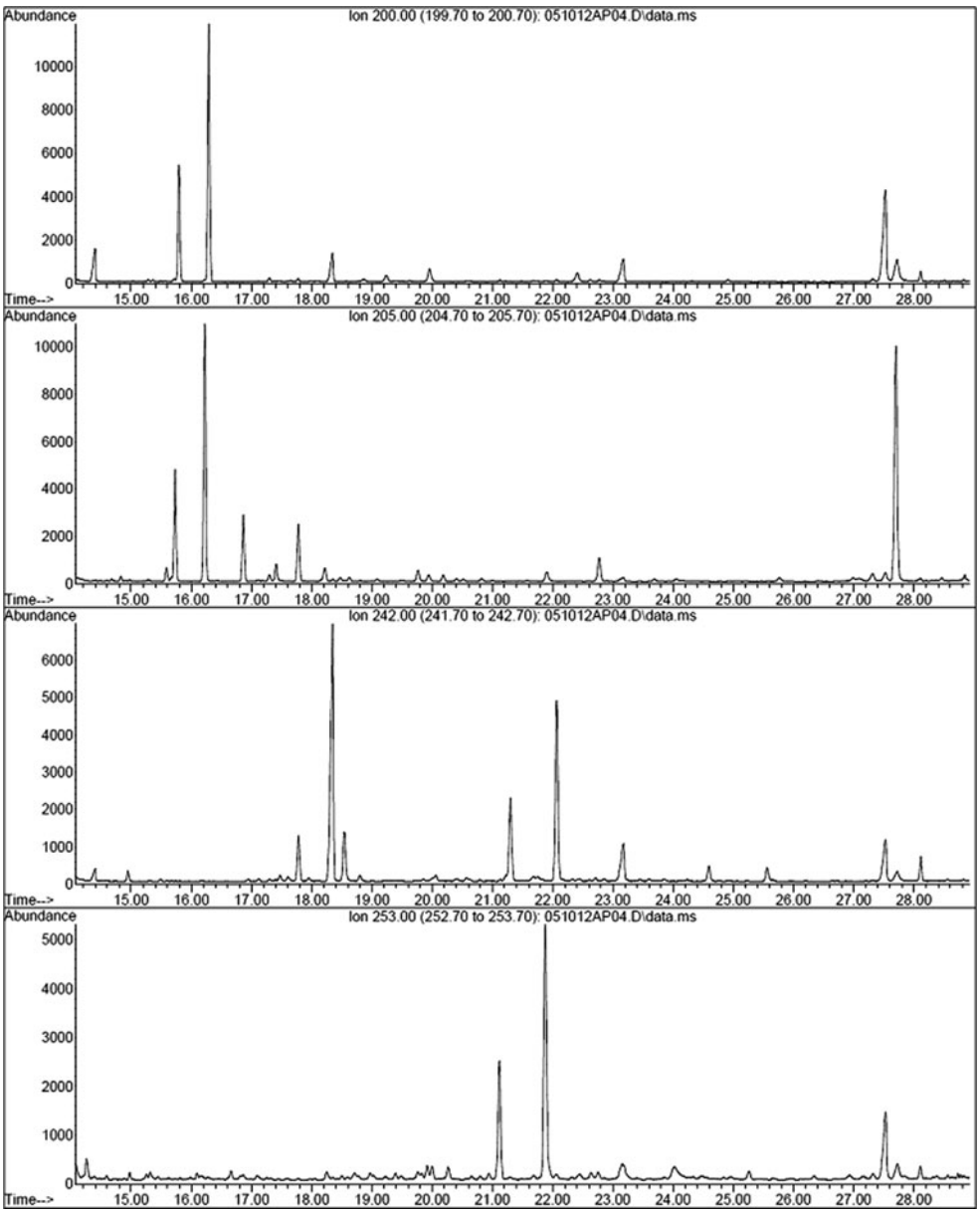

Fig. 2 Typical chromatograms of HHE *m*/*z* 200 retention time 15.81, 16.33 min; HHE-*d5*, *m*/*z* 205 retention time 15.74, 16.26 min; HNE, *m*/*z* 242 retention time 21.32, 22.10 min; and HNE-*d1*, *m*/*z* 253 retention time 21.13, 21.93 min. The chromatograms were obtained from oil samples spiked with 0.23 μg $g^{-1}$of HNE and HHE

Table 1 Statistical comparison of the addition curves with the calibration curves

		Addition curve		Calibration curve		*p* value
		Slope	R^2	Slope	R^2	
HNE	Potato crisps	0.7431	0.9986	0.7696	0.9989	0.4257
	Beef	0.7696	0.9989	0.7418	0.9940	0.2809
	Oil	0.7643	0.9929	0.6940	0.9995	0.0153
	Infant formula	0.6928	0.9909	0.5873	0.9625	0.0818
HHE	Potato crisps	1.0691	0.9984	1.1277	0.9994	0.0011
	Beef	1.1963	0.9977	1.1504	0.9979	0.0842
	Oil	1.1277	0.9994	1.1640	0.9970	0.1694
	Infant formula	1.0932	0.9966	1.2053	0.9984	0.0003

p values below 0.05 indicate statistically significant difference between the two slopes

Results and Discussion

Initial experiments were carried out in order to obtain the mass spectra and the retention times of the HNE, HHE, and the deuterated isotopic oximes. The corresponding mass spectra are shown in Fig. 1. The mass spectra of HNE are in agreement with those published from LaFond et al. (2011). All the oximes share the following ions *m*/*z* 73, *m*/*z* 181, and *m*/*z* 352, along with the characteristic ions *m*/*z* 242 for HNE, *m*/*z* 200 for HHE, *m*/*z* 253 for HNE *d11*, and *m*/*z* 205 for HHE *d5*. From the shared ions, *m*/*z* 352 was chosen for monitoring the compounds in SIM along with the characteristic ion of each analyte. The ratios of the common (*m*/*z* 352) and the characteristic ion in combination with the retention times were used for positive identification of the analytes. HNE, HHE, and their corresponding isotopes eluted as two peaks, since two stereoisomers were formed during the derivatization. Figure 2 illustrates typical chromatograms of each analyte and IS obtained at SIM during the analysis of oil samples spiked at 0.23 μg g^{-1} with HNE and HHE. Quantification was based on the sum of the two peak areas of the corresponding characteristic ion of each compound. Calibration and addition curves were constructed by plotting the ratio of the area of the analyte to the area of the isotope against the ratio of the mass of the analyte to the mass of the isotope. Knowing the exact mass of IS added in the sample prior to extraction, the mass of the analyte can be calculated.

In preliminary experiments, we have tried to derivatize HNE and HHE with PFBHA prior to the extraction as it has been reported in the literature (Michalski et al. 2008) in various food matrices. In the later study, the authors have reported the use of relatively high amount of PFBHA per analyzed sample. Due to the high cost of the reagent, we have tried to reduce the amount of reagent to the level of 20 mg per sample for direct derivatization in the matrix; however, this approach was proven unsuccessful since no aldehyde–PFBHA adducts could be detected. Thus, extraction of the analytes was considered necessary.

Table 2 HNE and HHE recoveries from the spiked matrices (n=4)

Matrix	HNE HHE added, μg g^{-1}	HNE detected, μg g^{-1}	SD	% CV	% Recovery	HHE detected, μg g^{-1}	SD	% CV	% Recovery
Potato crisps	0.72	0.71	0.06	9.1	99.0	0.66	0.01	1.7	93.2
	0.57	0.57	0.08	14.0	99.9	0.52	0.02	4.8	92.5
	0.39	0.38	0.02	4.9	95.6	0.36	0.01	3.7	91.2
	0.24	0.22	0.01	2.2	92.8	0.21	0.00	3.4	89.2
	0.08	0.09	0.01	10.5	113.5	0.08	0.00	1.5	107.2
Beef	0.37	0.37	0.04	11.1	98.0	0.37	0.06	15.3	98.1
	0.28	0.26	0.03	11.0	91.9	0.26	0.05	17.5	94.1
	0.20	0.19	0.01	5.1	90.4	0.19	0.02	11.2	94.7
	0.12	0.11	0.01	3.9	89.5	0.11	0.01	10.7	92.4
	0.04	0.04	0.01	13.0	89.6	0.03	0.00	11.3	90.6
Oil	0.71	0.74	0.04	5.9	104.3	0.76	0.01	1.8	99.0
	0.54	0.59	0.06	10.4	108.0	0.55	0.03	5.1	101.1
	0.37	0.36	0.02	7.0	97.7	0.38	0.01	3.1	100.6
	0.23	0.22	0.02	10.1	95.5	0.21	0.01	4.9	90.6
	0.08	0.07	0.02	22.4	90.0	0.07	0.01	19.6	85.5
Infant formula	0.72	0.62	0.05	12.9	87.0	0.66	0.03	5.0	92.7
	0.53	0.46	0.01	14.2	87.1	0.49	0.02	5.0	93.4
	0.40	0.36	0.06	16.3	89.8	0.39	0.03	8.0	96.5
	0.23	0.20	0.03	14.2	86.7	0.22	0.02	7.5	95.2
	0.08	0.07	0.01	18.3	87.2	0.08	0.02	23.7	107.6

Table 3 HNE and HHE recoveries from the spiked oil samples (n=6)

Matrix	HNE HHE added, μg g^{-1}	HNE detected, μg g^{-1}	SD	% CV	% Recovery	HHE detected, μg g^{-1}	SD	% CV	% Recovery
Oil	0.71	0.72	0.04	5.9	101.6	0.69	0.03	3.8	96.5
	0.55	0.57	0.06	9.8	104.9	0.54	0.03	5.9	99.0
	0.38	0.37	0.02	6.3	98.1	0.37	0.01	2.9	97.9
	0.24	0.23	0.03	11.6	97.1	0.22	0.01	4.5	91.5
	0.08	0.07	0.02	20.2	93.9	0.07	0.01	15.5	87.8

As mentioned previously, recovery of the analytes during the extraction can be highly influenced by the nature of the sample. Addition of HNE *d11* and HHE *d5* can eliminate underestimation of the analytes and minimize the variation between samples. However, high actual recoveries of the analyte increase the sensitivity of the method. Extraction of the analytes from oils with a mixture of water and methanol at proportions of 60/40 (*v*/*v*) and vortexing for 2 min was found to be efficient for actual recovery of HNE and HHE 75 and 85 %, respectively, at spiking levels between 0.1 and 1 μg g^{-1}. This was found remarkably less time consuming than what has been published by others (Surh and Kwon 2002; LaFond et al. 2011). The same proportion of methanol was chosen as extraction mixture for samples such as meat and potato crisps. However, when this extraction technique was applied on infant formula and other dairy products, the formed oximes could not be further extracted with pentane due to the incomplete separation of the aqueous and organic solvent phase which most probably occurred due to the presence of dairy proteins. In order to overcome this limitation, the QuEChERS method (Anastassiades et al. 2003) for determination of pesticide residues in fruits and vegetables was selected for the extraction of the analytes. In preliminary experiments, HNE along with HHE and their deuterated analogs were added in aqueous solutions of beef and infant formula and incubated at room temperature overnight. The analytes were extracted from the meat with a mixture of water and methanol 60/40 (*v*/*v*) and from the infant formula using the QuEChERS method. The amount of the analytes recovered was equal to the blank samples and notable low recovery of the IS was observed. α,β-Unsaturated aldehydes are known to form Schiff bases with the ε-NH_2 groups of lysine and Michael adducts between the electrophilic double bond and the nucleophilic groups of cysteine, histidine, and lysine (Uchida and Stadtman 1992; Rauniyar and Prokai 2009; Naveena et al. 2010).

In order to avoid interactions of the proteins present in the food samples with the IS and to ensure the recovery of the free aldehydes present, the extraction from meat samples was carried out with 60/40 (*v*/*v*) 1.66 M H_2SO_4/methanol. The acid present in the extraction mixture is expected to minimize the reactivity of the nucleophilic groups of the amino acids towards the analytes and the IS. For the extraction of the analytes from infant formula using the QuEChERS method, addition of acid was not found to be necessary as far as the extraction was carried out as soon as the IS was added. This could be attributed to alterations of the proteins and thus reduced reactivity with the aldehydes.

The matrix effect on the recovery of the analytes was evaluated by comparing the slopes of the calibration curves with those of the addition curves. A slight difference in the polarity of HNE compared to HNE *d11* due to the high level of deuterium atoms can be expected. Thus, calibration curves were prepared by applying the corresponding extraction method for each matrix excluding the sample. However, apart from the

Table 4 LOD and LOQ (nanogram per gram of sample) for the different matrices

		Potato crisps	Beef	Oil	Infant formula
HNE	LOD	20.1	4.2	17.2	32.1
	LOQ	61.2	12.8	52.1	97.3
HHE	LOD	7.3	4.2	10.4	9.4
	LOQ	21.2	12.7	31.1	28.4

Table 5 HNE and HNE levels in analyzed food samples. n=4 whereas ±standard deviation is mentioned. The rest are obtained from a single analysis of each sample.

Sample description	HNE, ng g^{-1} sample	HHE, ng g^{-1} sample
Frying oil ω-3 enriched	3,750±24	520±10
Frying oil	3,760±20	181±5
Infant formula	209±10	21±3
Sardines canned	Not detected	25±4
Colza oil	111	149
Extra virgin olive oil A	69	155
Extra virgin olive oil B	117	141
Extra virgin olive oil C	57	99
Peanuts A	598	Not detected
Peanuts B	124	Not detected
Cookies A	132	25
Cookies B	103	23
Cookies C	179	21
Walnuts A	136	24
Walnuts B	406	33

difference in polarity between HNE and the deuterated analogue, chemical interactions with the matrix due to the aldehyde group, double bond, and hydroxyl group are expected to be identical. The results of the statistical evaluation of the slopes of the calibration and addition curves are shown in Table 1. Concerning HNE, only the oil samples appear to have a slight but statistically significant matrix effect (p=0.0153). In the case of HHE, a matrix effect is evident in potato crisps and infant formula (p=0.0011, p=0.0003).

The trueness of the measurement was assessed through the recovery of the analytes added at different levels (addition curves) as suggested by the European Union Commission decision 2002/657/EC (European Community 2002) and the results are illustrated in Table 2. The values are the mean of four replications of analysis performed in two different days. Concerning HNE, the results indicate that in all the matrices the error from the actual amount added in the sample is, with the exception of the recovery from potato crisps at the lower spiked level, within 86.7 and 108.0 %. For HHE, recoveries are between 85.5 and 107.6 %. This is within the limits of the directive for concentrations above 10 μg g^{-1} (−20 to +10 %). The data suggest that the matrix effect, indicated by the comparison of the slopes of the addition and calibration curves, did not have a significant impact on the accuracy of the method. In order to examine the repeatability, different samples of each group were analyzed in duplicate in two different days and the coefficient of variation (CV) was found to be below 20 % for both analytes in four different matrices with the exception of HNE in oil samples at concentration of 80 ng g^{-1} (CV 22.4 %) and HHE in infant formula samples at concentration of 79 ng g^{-1} (CV 23.7 %). Furthermore, reproducibility was evaluated by analyzing oil samples using two different analysts in different days and the results are shown in Table 3. Regarding HNE, the CV varied between 5.9 and 20.2 % while for HHE between 2.9 and 15.5 %.

Limits of detection (LOD) and limits of quantification (LOQ) were calculated based on the standard deviation (SD) of the residuals of the response and the slope of the addition curve (Mocak et al. 1997). LOD equals to 3.3×(SD/Slope), while LOQ equals to 10×(SD/Slope). LODs and LOQs were determined for each matrix and the results are shown in Table 4. Statistical determination of the LODs and LOQs is indicating higher values compared to empirical methods, i.e., methods based on the calculation of the signal-to-noise ratio. Based on the available results in the literature (Surh and Kwon 2005; Seppanen and Csallany 2001; Surh et al. 2007) together with the results from the samples analyzed with the present method, the LODs and LOQs are below the expected concentrations.

Table 5 illustrates the levels of HNE and HHE detected in selected samples available in the Belgian market. As already mentioned, HNE is expected to be formed during oxidation in foods containing ω-6 fatty acids, while formation of HHE is related to the presence of ω-3 fatty acids. In fact in samples such as peanuts where the dominating PUFA is linoleic and ω-3 PUFAs are not present, only HNE could be detected. In walnuts where linolenic acid was found to be in average 11 % of the fatty acids, HHE was detected but in significantly lower levels than HNE. Extra virgin olive oils with an average fatty acid composition of 78 % oleic, 4.5 % linoleic, and 0.7 % linolenic demonstrated higher amounts of HHE as compared to HNE, indicating that linolenic acid was more prone to oxidation. Heating of the oils at temperatures applied in frying resulted in formation of significant amounts of HNE and HHE indicating that consumption of fried foods can contribute to high intake of these aldehydes. The levels of HNE and HHE in the frying oils before heating were below the LODs.

Conclusions

We have designed, implemented, and validated an accurate and reproducible analytical technique for determination of free HNE and HHE that can be applied in a broad range of food samples. The results are not affected by the analyzed matrix and are not dependent on the recovery of the analyte during the extraction. We suggest that milk and dairy products or generally samples containing surface active compounds can be analyzed with the QuEChERS method. The method in which the analytes are extracted with acidified methanolic solution developed and validated on raw beef and crisps could also be applied on cooked meat and meat products, dry nuts, and cookies. Oils, butter, margarines, and mayonnaise could be analyzed by the same method.

Acknowledgments The authors gratefully acknowledge the Federal Public Service of Health, Food Chain Safety and Environment (project RT/10 TOXAL) for funding this research.

Compliance with Ethics Requirements Antonios Papastergiadis has received a research grant from the Belgian Federal Public Service of Health.

Conflict of Interest Edward Mubiru does not have a conflict of interest. Herman Van Langenhove does not have a conflict of interest. Bruno De Meulenaer does not have a conflict of interest. This article does not contain any studies with human or animal subjects.

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