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Comparative evaluation of methods for the detection of 2-alkylcyclobutanones as indicators for irradiation treatment of cashew nuts and nutmeg

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ABSTRACT

Irradiation of food products and ingredients must be indicated by proper labeling. This study evaluated the appropriateness of the European Standard EN 1785:2003 for the detection of 2-alkylcyclobutanones, which are radiolysis products of fatty acids, in cashew nuts and nutmeg and confirmed its suitability to detect irradiation of cashew nut samples at average absorbed doses of 1 kGy and above. An alternative method was developed, which is based on matrix solid phase dispersion and subsequent separation and detection of oxime derivatives of 2-alkylcyclobutanones by high performance–high resolution mass spectrometry. It is more rapid, less resource consuming, and more sensitive than EN 1785:2003. This method allowed detection of 2-alkylcyclobutanones in cashew nuts irradiated at 100 Gray and in nutmeg irradiated at 400 Gray. None of the 26 cashew nut and 14 nutmeg samples purchased in different EU Member States contained traces of 2-alkylcyclobutanones.

1. Introduction

Food irradiation is approved for use in over 60 countries for various applications and purposes in a wide variety of foodstuffs, mostly as a post-harvest phytosanitary measure. The irradiation of certain foods and food ingredients is regulated in the EU by Directive 1999/2/EC (European Communities, 1999a). The Community list of foodstuffs which may be treated with ionizing radiation to the exclusion of all others and the maximum radiation doses authorized are given in Directive 1999/3/EC (European Communities, 1999b). The only harmonized entry at EU level is for dried aromatic herbs, spices and vegetable seasonings at a maximum overall average absorbed radiation dose of 10 kGy. However, authorisations at the level of EU Member States exist for a wider variety of foods (European Union, 2009). Proper labeling of irradiated food products and ingredients is required at EU level as well as by the FAO/WHO Codex Alimentarius (Codex Alimentarius, 2003). For checking compliance with legislation a number of analytical methods have been elaborated and standardized by the European Committee for Standardization (CEN). Among this suite of methods is EN 1785:2003 Foodstuffs – Detection of irradiated food containing fat – Gas chromatographic/mass spectrometric analysis of 2-alkylcyclobutanones (European Committee for Standardization, 2003). The standard specifies a method for the identification of irradiation treatment of food containing fat. It is based on the mass spectrometric (MS) detection of radiation-induced 2-alkylcyclobutanones (2-ACBs) after gas chromatographic (GC) separation. The method has been successfully tested in inter-laboratory trials on raw chicken, pork, liquid whole egg, salmon and Camembert. Other studies demonstrate that the method is applicable to a wide range of foodstuffs, although for mangoes, a small number of false positives were reported. However, these were attributed to analytical difficulties encountered as 2-ACBs have never been detected in non-irradiated samples of this product.

A study by Variyar, Chatterjee, Sajilata, Singhal, and Sharma (2008) claimed the natural occurrence of 2-ACBs in cashew nut (*Anacardium occidentale*) and nutmeg (*Myristica fragrans*), thus disproving the hypothesis that 2-ACBs are radiolytic degradation

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products of fat which can serve as unique markers for irradiation treatment of fatty foods. The authors postulated that a special extraction technique, i.e. supercritical fluid extraction (SFE) using carbon dioxide, in combination with clean-up of the extract using thin-layer chromatography allowed them to identify traces of 2-ACBs in the mentioned food products. For nutmeg those findings have not been confirmed by another independent study, although a similar analytical approach (SFE with SPE clean-up) was applied (Chen et al., 2012). Meanwhile, other reports came out supporting the fact that 2-ACBs do not occur naturally (Leung, Tang, Ye, & Chan, 2013; Driffield et al., 2014). The latter reports did not use EN 1785 for the determination of 2-ACBs but claimed that their analytical methodology is superior or at least equivalent.

The methodology used in EN 1785:2003 has been developed and validated in the 1990s (Raffi et al., 1994). Since then enormous progress has been made in analytical technologies and instrumentation, allowing miniaturization of solvent volumes and improvements in sensitivity for the analysis of 2-ACBs (reviewed by Crews, Driffield, and Thomas (2012)). For example, proposals have been made to replace Soxhlet extraction by pressurised liquid extraction (PLE) to isolate total fat (Obana, Furuta, & Tanaka, 2005), to apply a direct extraction method using acetonitrile (Hijaz, Kumar, & Smith, 2010), to use gel-permeation chromatography instead of Florisil for clean-up and GC–MS/MS instead of GC–MS (Takahashi, Ishii, & Matsumoto, 2013), or replacing GC–MS by LC–MS/MS (Leung et al., 2013; Driffield et al., 2014). Next to these methods of analysis using mass spectrometric detection approaches based on ELISA (Zhao, Wang, Li, & Ha, 2013) and biosensors (Zhao, Ha, Yue, & Wang, 2015) also exist.

Triggered by the report that 2-ACBs might not be unique indicators of irradiation we compared the merits of several novel approaches for the analysis of 2-ACBs in cashew nuts and nutmeg using the performance of EN 1785:2003 as benchmark. In addition, cashew nut and nutmeg samples purchased in different EU Member States were assessed to verify the assumption that 2-ACBs do not occur in non-irradiated foods.

2. Materials and methods

2.1. Chemicals

2-Dodecylcyclobutanone (2-DCB), 2-tetradecylcyclobutanone (2-TCB), aceton-D_6 ($C_3{}^2H_6O$), methanol-D_1 (CH_3O^2H), sodium deuterium oxide (NaO^2H), heavy water (^2H_2O), deuterium chloride (^2HCl), formic acid (LC–MS grade), and Florisil (PR grade 60–100 mesh) were purchased from Sigma–Aldrich (St. Louis, USA). All solvents were of chromatographic grade, either GC or HPLC, and were purchased from VWR with the exception of methanol (MeOH), isopropanol (iPrOH), and water (mobile phase) which were of LC–MS grade and purchased from Fluka (Sigma–Aldrich, St. Louis, USA).

2-DCB and 2-TCB were delivered in vials containing a nominal amount of 5 mg each. The content of the vials was dissolved in *n*-hexane and quantitatively transferred to 5 mL volumetric flasks which were made up to the mark with *n*-hexane. This resulted in solutions of nominally 1 mg/mL 2-DCB and 1 mg/mL 2-TCB in *n*-hexane. From these two stock solutions mixed working solutions of different concentrations were prepared.

2.2. Stable-isotope labeled internal standard

To facilitate the control of extraction, clean-up, and measurement a stable-isotope labeled analoge of 2-DCB was synthesised in-house. Under very strong alkaline conditions the three hydrogens in the positions 2,4,4 of 2-DCB were exchanged against deuterium to give 2,4,4-$^2H_3$-2-dodecylcyclobutanone (D_3-2-DCB).

In brief, 53 mg of 2-DCB were dissolved in a mix of 1 mL aceton-D_6 ($C_3{}^2H_6O$) and 10 mL methanol-D_1 (CH_3O^2H). 610 μL of 14 mol/L sodium deuterium oxide (NaO^2H) in heavy water (^2H_2O) were added, the reaction vessel was evacuated to a pressure of ca. 150 mbar, and incubated at 60 °C for 24 h. After cooling to room temperature 11 mol/L deuterium chloride (^2HCl) in heavy water (^2H_2O) were added until neutral pH. The reaction mixture was then evaporated to dryness at 60 °C under nitrogen atmosphere and suspended in 2,2,4-trimethylpentane. The organic phase was washed three times with water and then dried over sodium sulfate (Na_2SO_4). After filtration of the organic phase it was evaporated to dryness under vacuum.

The reaction product was cleaned up by preparative reversed-phase HPLC. A Shimadzu LC20 AD solvent delivery system with a low pressure gradient unit delivered a flow of 4 mL/min of MeOH/ i-PrOH/ H_2O (63/27/10, v/v/v) to a Supelco Discovery HS C18 250 × 10 mm, 5 μm, column (Sigma–Aldrich, St. Louis, USA). 50 μL/min of the effluent were split off, made up to a flow of 300 μL/min with the same mobile phase, and fed into an Orbitrap Elite mass spectrometer with APCI source to monitor for D_3-2-DCB in single MS high resolution mode. The fraction of the remaining flow containing D_3-2-DCB was collected. This fraction was concentrated and injected onto a Supelco Discovery HS F5 250 × 10 mm, 5 μm, column (Sigma–Aldrich, St. Louis, USA) for additional clean-up under the same conditions as above.

From the final cleaned up product a solution of D_3-2-DCB equivalent to app. 200 μg 2-DCB per mL of *n*-hexane was prepared and used throughout the whole study.

2.3. Food irradiation

To investigate the effect of irradiation on cashew nut and nutmeg, two samples of each were selected and prepared as above. Each material was subdivided in two for a total of four subsamples of either cashew nut or nutmeg. Those subsamples were then irradiated with gamma radiation at an average absorbed dose of 100, 400, 700, and 1000 Gy at the Helmholtz Zentrum Berlin, Germany. For dosimetry a PTW Unidose E in connection with a semiflex chamber type 31013 (PTW, Freiburg, Germany) was used.

For purposes of method development and verification, a cashew nut and a nutmeg sample were used which were irradiated at a very high dose between 8.6 and 10.9 kGy at a different facility specialized in food irradiation (SynergyHealth, Etten-Leur, The Netherlands). Those highly irradiated samples were used to verify that EN 1785:2003 and all the developed alternative methods were indeed able to detect the 2-ACBs.

2.4. Cashew nut and nutmeg samples

Samples (pre-packaged) were obtained from retail outlets in a number of EU Member States (Austria, Belgium, Czech Republic, Germany, Spain, Croatia, Hungary, Italy, Romania, United Kingdom). None of the samples were labeled as being irradiated. A number of samples were obtained in stores operated by international corporations; therefore, it cannot be guaranteed that all samples came from different wholesalers, although the chance that they came from the same lot is rather low.

The samples were stored at room temperature and prepared as follows for analysis: about 10 to 20 g of whole cashew nuts were shock frozen with liquid nitrogen. The frozen nuts were then transferred to a knife mill Grindomix GM200 (Retsch, Haan, Germany) and comminuted for 5 s at full speed. This resulted in a fine powder for most of the Cashew nut samples with exception of 8 salted and roasted samples for which more paste-like materials were obtained.

Seven of the nutmeg samples were already ground and not treated any further. For the other 7 samples of whole nutmeg kernels were first crushed, then shock frozen with liquid nitrogen and comminuted as above. This resulted in a powder with slightly larger particles then the already ground nutmeg.

2.5. Methods

Fig. 1 provides an overview of the analytical techniques used in this study. The European Standard EN 1785:2003 served as a benchmark to assess whether alternative methods were more selective and/or more sensitive for the detection of 2-ACBs in cashew nut and nutmeg.

2.5.1. EN 1785:2003

The European Standard EN 1785:2003 was used with the following minor modifications: the internal standard 2-cyclohexylcyclohexanone was replaced by D_3-2-DCB which was added to the fat extract before clean-up to control clean-up efficiency. Furthermore, methyl pentadecanoate and methyl heptadecanoate were added to the injection solution after clean-up to have additional control about possible retention time shifts; an automated Soxhlet extractor Büchi B-811 (Büchi, Flawil, Switzerland) was used for fat extraction; wash and elution volumes for the Florisil column chromatography were optimized and reduced to 80 mL each instead of the described 150 mL each. To minimize environmental impact the *n*-hexane used during analysis was recycled as much as possible.

The GC–MS consisted of an Agilent 6890 gas chromatograph connected to an Agilent 5973 inert mass spectrometer (Agilent, Santa Clara, USA). Separation was afforded with a DB-5MS column (28 m, 0.25 mm I.D., 0.25 μm film; J&W Scientific, Agilent, USA) in constant flow mode with 0.6 mL/min helium. The temperature program was: 70 °C initial, 1 min hold, 20 °C/min to 270 °C, 40 °C/min to 350 °C, 2 min hold. The injector was used in splitless mode at 250 °C with a purge time of 0.5 min and a purge flow of 100 mL/min.

Because identification with GC–MS requires a reference material for comparison and only 2-DCB and 2-TCB are easily accessible EN 1785:2003 allows only the identification of those substances.

2.5.2. Alternative extraction methods

To compare yields of different extraction approaches a cashew nut sample, blank of 2-ACBs, was mixed at three different ratios with the highly irradiated cashew nut sample. The same was done for nutmeg. The mixing ratios were: 5 g blank plus 0 g irradiated, 4.5 + 0.5, and 4 + 1. Of these test sets one was extracted according to EN 1785:2003 with *n*-hexane, one with pressurized liquid extraction (PLE) and ethyl acetate, one with PLE and acetonitrile (ACN), and one with ultrasound assisted extraction (UAE) and ACN (Fig. 1).

For PLE the test portion was mixed with an equal amount of sodium sulfate and filled into a 34 mL capacity extraction vessel. The remaining empty space was filled up with sand. The settings for the PLE were as follows: extraction temperature 100 °C, extraction pressure 1500 psi, preheating 5 min, static extraction 5 min, solvent flush 10 mL, and nitrogen purge 60 s.

For UAE the test portion was suspended in 10 mL ACN and then sonicated for 30 min (Bandelin Sonorex, Berlin, Germany). After a brief spin in a centrifuge to pellet particulate matter at the bottom

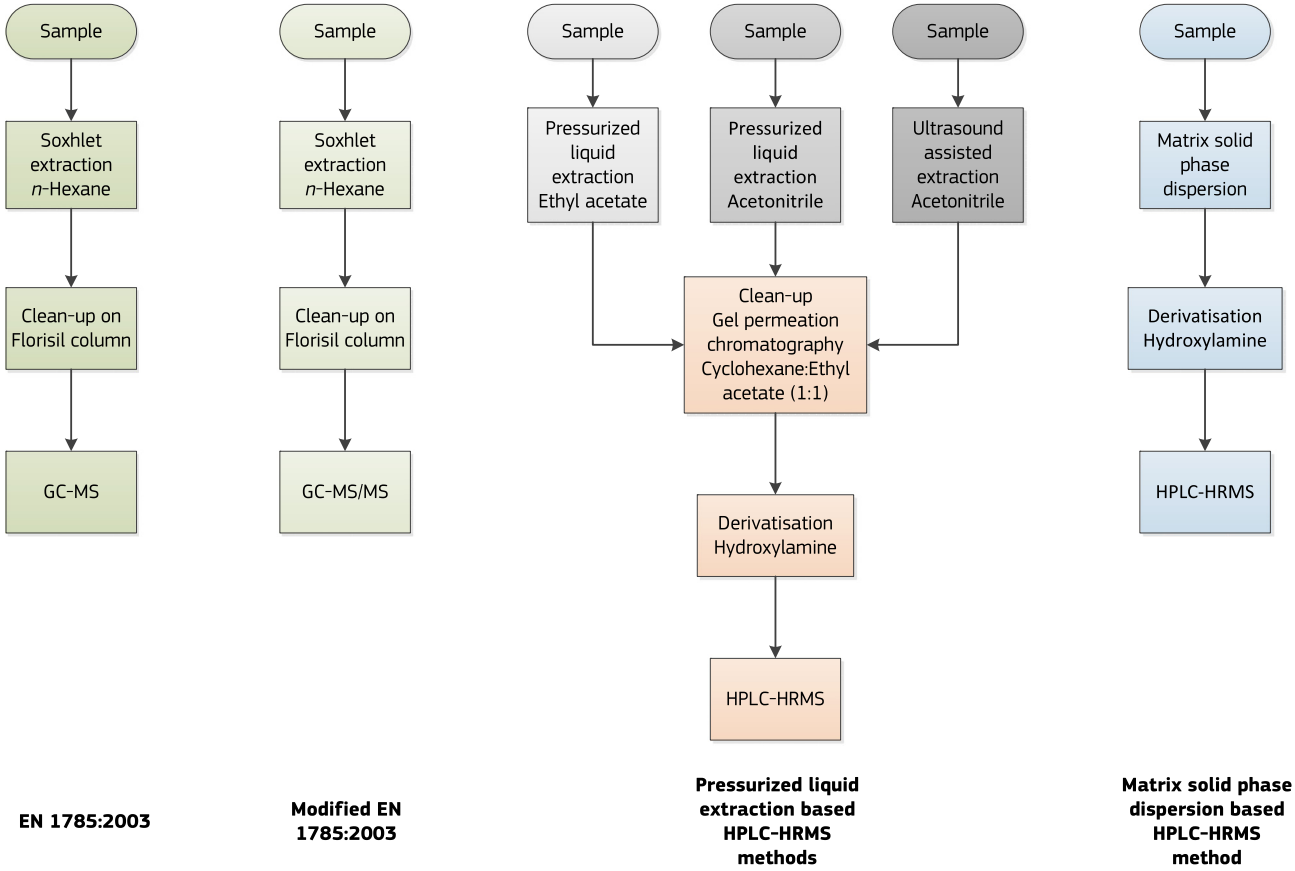

Fig. 1. Overview of applied measurement techniques for the determination of 2-ACBs in cashew nut and nutmeg samples (HPLC–HRMS: high-performance liquid chromatography–high resolution mass spectrometry; GC–MS: gas chromatography–mass spectrometry; GC–MS/MS: gas chromatography–tandem mass spectrometry).

of the tube the supernatant was transferred to a second tube. This process was repeated twice and all supernatants were combined.

The extracts of the different extraction approaches were dried under vacuum at 40 °C with a Laborota 4001 rotary evaporator (Heidolph, Schwabach, Germany) and diluted with cyclohexane/ethyl acetate (50/50, v/v) to 0.2 g/mL or, if less than 1 g of dry residue were present, to a total volume of 2.5 mL.

For matrix solid phase dispersion (MSPD) 0.5 g sample and 1 g C18 solid phase material (Discovery DSC-18, Supelco, USA) were mixed for 3 min in a small mortar. The mix, which had a homogenous appearance, was then transferred into an empty 6 mL polyethylene syringe barrel with a polyethylene frit at the bottom. A second frit was inserted on top of the mix and then compressed to a bed height of ca. 1.5 cm. These cartridges were then eluted with 3 × 3 mL ACN. The eluate was collected in a single tube and taken to dryness under vacuum at 40 °C with a Genevac EZ-2 plus centrifugal vacuum evaporator (Genevac Ltd., Ipswich, UK).

2.5.3. Gel permeation chromatograph (GPC) clean-up

The extracts of the different extraction approaches were loaded onto a GPC column 33 cm length × 2.5 cm I.D. filled with BioBeads SX-3 (BioRad, Hercules, USA) using a Valco Cheminert six-port, two-position low pressure valve (VICI AG International, Schenkon, Switzerland) with a 5 mL sample loop and eluted with cyclohexane/ethyl acetate (EtOAc) (50/50, v/v) at a flow rate of 5 mL/min, which was delivered by a LC-20 AD pump (Shimadzu, Kyoto, Japan). The fraction of 90 to 120 ml elution volume, containing the 2ACBs, was collected and taken to dryness under vacuum at 40 °C with a Laborota 4001 rotary evaporator.

2.5.4. Oximation of 2-ACBs

Dry sample extracts or calibration solutions were reconstituted with 200 µL ACN. 50 µL of those reconstituted extracts were then mixed with 50 µL of 27.8 mg hydroxylamine per milliliter of ACN/H_2O (50/50, v/v) (Ye, Liu, Horvatovich, & Chan, 2013). After a minimum of 60 min at room temperature the mixtures containing the 2-ACB-oximes were inject without any further processing.

2.5.5. Detection of 2-ACBs by gas chromatography–tandem mass spectrometry (GC–MS/MS)

The GC–MS/MS consisted of an Agilent 6890 GC connected to a Micromass Quattro Micro GC (Micromass/Waters, Manchester, UK). The MS was operated in positive chemical ionization mode with methane as reactant gas. Separation was afforded by a DB-1 column (15 m, 0.25 mm I.D., 0.25 µm film; J&W Scientific, Agilent, USA) with the following temperature program: 55 °C initial, 1 min hold, 15 °C/min to 300 °C, 5 min hold, in constant flow mode of 1 mL/min He. An injection solution volume of 1 µL was injected into the injector at 250 °C in splitless mode with a purge time of 0.5 min and a purge flow of 100 mL/min. Data were acquired in selected reaction monitoring with the analyser settings shown in Table 1.

2.5.6. Detection of 2-ACB oximes by high-performance liquid chromatography–high-resolution mass spectrometry (HPLC–HRMS)

The HPLC–HRMS instrument consisted of two LC20 AD solvent delivery systems for a binary high-pressure gradient, a SIL 30 AC auto-sampler, a CTO 30 A column oven (Shimadzu, Kyoto, Japan), and an Orbitrap Elite mass spectrometer (Thermo Scientific, Bremen, Germany). Chromatographic separation was afforded by an Ascentis express Phenyl column (100 × 2.1 mm, particle size 2.6 µm; Supelco, USA) with mobile phase A: H_2O/formic acid (999/1, v/v) and mobile phase B: ACN/iPrOH/formic acid (800/199/1, v/v/v). The following gradient conditions were used: 0 min 45% B, 8 min 100% B, 13 min 100% B, 13.01 min 45% B, 15 min 45% B with a flow rate of 300 µL/min.

Table 1
Selected reaction monitoring parameters for GC–MS/MS measurements of 2-ACBs.

Analyte	Precursor ion [Da]	Product ion [Da]	Dwell time [s]	Collision energy [eV]	Delay [s]
2-DCB	239.2	95.1	0.05	7	0.02
	239.2	109.1	0.05	7	0.02
D_3-2-DCB	242.2	98.1	0.05	7	0.02
	242.2	112.1	0.05	7	0.02
2-TCB	267.2	95.1	0.05	7	0.02
	267.2	109.1	0.05	7	0.02

The effluent of the chromatographic column was ionized with heated electro spray with the following source settings: vaporizer temperature 300 °C, ion transfer tube 275 °C, sheath gas 30 arbitrary flow units (afu), auxiliary gas 15 afu, sweep gas 2 afu, s-lens 67.7, and spray voltage 3 kV. Skimmer offset voltage was set to 10 V to provide some declustering in the source region. Data acquisition consisted of one scan ranging from *m/z* 200 to 400 with resolving power 15,000. This was followed by a product ion scan with Higher-Energy Collisional Dissociation (HCD) fragmentation at resolving power 15,000, normalized collision energy 60, and isolation width 5 amu. The following precursor masses were selected: *m/z* 226.2165, *m/z* 255.7500, and *m/z* 281.2635.

The presence of a common product ion with *m/z* 82.0651 (C_5H_8N; for D_3-2-DdCB *m/z* 85.0840, $C_5{}^1H_5{}^2H_3N$), which fully preserves the 2-substituted cyclobutanone structure, was seen as specific indicator for the presence of 2-ACBs. The full scan data was used to confirm molecular mass and elemental composition.

3. Results and discussion

3.1. Verification of the performance of EN 1785:2003 for cashew nut and nutmeg

According to EN 1785:2003, samples are considered to be irradiated when at least one of the two targeted 2-ACBs is present at a level yielding a signal to noise ratio of at least 3 to 1 (detection limit) using the least sensitive ion.

The applicability of the method was tested by analyzing cashew nut and nutmeg samples that were treated with low doses of ionizing irradiation (100, 400, 700 and 1000 Gy) to estimate what level of irradiation could be detected by the method. It was confirmed that EN 1785:2003 is applicable for the detection of low-level irradiation doses in cashew nuts; 2-DCB was clearly detected in a sample irradiated at 700 Gy. The absolute detection limits for 2-DCB and 2-TCB were 50 µg/kg cashew nut (for both compounds); related to crude fat the detection limit was about 100 µg/kg.

EN 1785:2003 was not able to detect irradiation at low doses (<1 kGy) in nutmeg. One reason for this deficiency might be the fact that nutmeg fat ("nutmeg butter"), whose main component is trimyristin, is solid at room temperature and has limited solubility in *n*-hexane of room temperature. This led repeatedly to partial crystallization of the nutmeg fat in the upper part of the Florisil column, which might have negatively impacted the clean-up efficiency.

Another reason for this deficiency could be that neither 2-DCB nor 2-TCB, the two markers targeted by EN 1785:2003, was the most abundant marker of irradiation in nutmeg. The main fatty acid occurring in nutmeg butter is myristic acid whose radiolysis product is 2-decylcyclobutanone. Since the latter will also produce the same mass fragments as the other two 2-ACBs, a prominent peak eluting in front of 2-DCB could be due to 2-decylcyclobutanone. Unfortunately, no reference substance was commercially available to verify this assumption. Anyhow, the

presence of 2-decylcyclobutanone in irradiated nutmeg was tentatively confirmed with another technique (see below).

3.2. Detection of 2-ACBs by gas-chromatography–tandem mass spectrometry (GC–MS/MS)

An attempt was made to improve sensitivity and selectivity of the procedure as described in EN 1785:2003 by replacing the mass spectrometric detector by a triple quadrupole mass spectrometer as suggested by Takahashi, Ishii, and Matsumoto (2013). The authors reported detection limits of 15 and 20 μg/kg fat isolated from beef patties using electron ionization (EI) at 70 eV. EI of 2-ACBs create mostly ions of *m*/*z* 98, which would not offer significant improvements in sensitivity as compared to single-quadrupole instruments. For this reason the ionization mode was changed to chemical ionization with methane as reactant gas to create meaningful precursor ions for further fragmentation and selected reaction monitoring (Table 1). Unfortunately, this modification of EN 1785:2003 did not lead to significant improvements of the method's sensitivity to detect irradiation. The main reason for this was possibly the fact that the ionization efficiency in chemical ionization is poorer than in electron ionization, which off-set the benefits of the improved selectivity of GC–MS/MS. Therefore, this technique was not further investigated.

3.3. Detection of 2-ACB oximes by high performance liquid chromatography–high-resolution mass spectrometry (HPLC–HRMS)

For 2-ACBs to be detected by mass spectrometry (MS) they need to be ionized. In GC–MS this happens, in the vast majority of applications, through electron ionization (EI). This is very robust and efficient but results in strong fragmentation. The resulting fragments lead to signals which possess little specificity for 2-ACBs. In HPLC-MS electro spray ionization (ESI) is the most commonly used source of ions. This is not very efficient for native 2-ACBs but the ionization efficiency of 2-ACBs can be significantly improved through a very simple derivatisation reaction leading to the formation of the respective oximes as proposed by Ye et al. (2013). Then very specific signals can be recorded with high sensitivity. Leung et al. (2013) have already applied this approach to check for the presence of 2-ACBs in cashew nut and nutmeg. Therefore, this approach was further used for testing which alternative extraction and clean-up procedures allow for the most sensitive detection of 2-ACBs while at the same time minimizing labor and time expenditure.

During the setup of the HPLC–HRMS method with the highly irradiated cashew nut and nutmeg materials unknown prominent peaks were seen in the extracted ion chromatogram of *m*/*z* 82.0651, which is highly selective for the 2-ACB oximes (Figs. 2 and 3). Since the profile of 2-ACBs formed by irradiation reflects the fatty acid profile of the irradiated food one of the unknown substances could be 2-decylcyclobutanone resulting from radiolysis of myristic acid and the other could be 2-tetradecenylcyclobutanone formed from oleic acid. Since no commercial sources for 2-decylcyclobutanone or 2-tetradecenylcyclobutanone reference materials could be found, the occurrence of those substances was tentatively identified by elemental composition, product ion spectrum, and retention time of the oxime in reversed-phase liquid chromatography. The elemental composition of 2-decylcyclobutanone oxime is $C_{14}H_{27}NO$ with an expected *m*/*z* 226.2165 of the protonated molecule ($[M+H]^+$). A value of *m*/*z* 226.2165 was measured. In the product ion spectrum the fragments with *m*/*z* of 82.0651, 96.0808, and 110.0964 were expected. The following fragments were measured: *m*/*z* 82.0646, *m*/*z* 96.0802, and *m*/*z* 110.0961. The retention time of the peak tentatively containing 2-decylcyclobutanone oxime was 3.63 min. Since 2-decylcyclobutanone oxime is a homologue of 2-DCB oxime (4.46 min) and 2-TCB oxime (5.18 min) the retention time differences between these peaks were expected to be very similar. The difference between 2-DCB oxime and 2-TCB oxime was determined to be 0.72 min. The retention time difference between the peak tentatively identified as 2-decylcyclobutanone oxime and 2-DCB oxime was 0.83 min. Because of the excellent agreement of the calculated *m*/*z* with the observed *m*/*z* of 2-decylcyclobutanone oxime and its fragments in the product ion spectrum and the consistency of the predicted retention time, the peak at 3.61 min was tentatively identified as 2-decylcyclobutanone oxime (Fig. 2, Panel a, Peak 3; full product ion spectrum in Supplementary material).

The elemental composition of 2-tetradecenylcyclobutanone oxime is $C_{18}H_{33}NO$ (*m*/*z* 280.2635 for $[M+H]^+$) with the same typical product ions of 2-ACB oximes. Because of the presence of a mono-unsaturation a retention time shorter than the corresponding fully saturated 2-TCB but longer than 2-DCB was expected. The following ions were measured for the peak with a retention time of 4.73 min: *m*/*z* 280.2636, 82.0648, 96.0806, and 110.0963. Therefore, the peak at 4.73 min was tentatively identified as 2-tetradecenylcyclobutanone (Fig. 2, Panel b, Peak 4; full product ion spectrum in Supplementary material).

3.4. Extraction of 2-ACBs

Even though recovery of 2-ACBs, spiked at low levels into cashew nut, was complete with the extraction/clean-up procedure prescribed in EN1785:2003, alternatives were investigated that would allow to extract incurred 2-ACBs, present as a result of low-level irradiation, to an equal extent without co-extracting much of the accompanying triglycerides.

As alternative methods for extraction (Fig. 1) pressurized solvent extraction (PLE) and ultrasound assisted extraction (UAE) were applied to selected samples with two different polar extraction solvents, EtOAc as proposed by Obana et al. (2005) and ACN as proposed by Hijaz et al. (2010).

PLE allowed the extraction to be done within 15 min and UAE within 30 min as opposed to the 6 h of the Soxhlet extraction of EN 1785:2003. Changing the extraction solvent from the very apolar *n*-hexane to the more polar EtOAc or ACN limited the co-extraction of lipids. These extracts were then cleaned up with gel permeation chromatography which allowed using more of the extracted fat. This was done with the aim of increasing the sensitivity of the method. For example, in the case of PLE with EtOAc an equivalent of ca. 2 g of cashew nut or nutmeg was loaded for clean-up. This even increased to the equivalent of 5 g in the case of PLE with ACN and UAE with ACN. The loading equivalent of the clean-up by Florisil column chromatography as prescribed in EN 1785:2003 was only 0.5 g. The cleaned up extracts were then measured with high-performance liquid chromatography–high resolution mass spectrometry (HPLC–HRMS).

For both, cashew nut and nutmeg, PLE with ACN achieved complete extraction within only 15 min extraction time and 50 mL solvent consumption. This was a clear improvement compared to the EN 1785:2003 approach. But still a 30 min clean-up per sample with GPC using 150 mL of organic solvent was involved.

In view of the successful application of ACN as extraction solvent and to further reduce total solvent and time consumption MSPD was investigated. Through proper choice of the solid phase, i.e. C18 material, and the elution solvent, i.e. ACN, a high extraction yield with little co-extraction was obtained.

Table 2 lists the performance characteristics in terms of recoveries and detection limits for cashew nut and nutmeg for EN 1785:2003, modified EN 1785:2003 (GC–MS replaced by GC–MS/MS) and HPLC–HRMS of 2-ACB oximes. To determine apparent recoveries and limit of detection 0.5 g of cashew nut and nutmeg

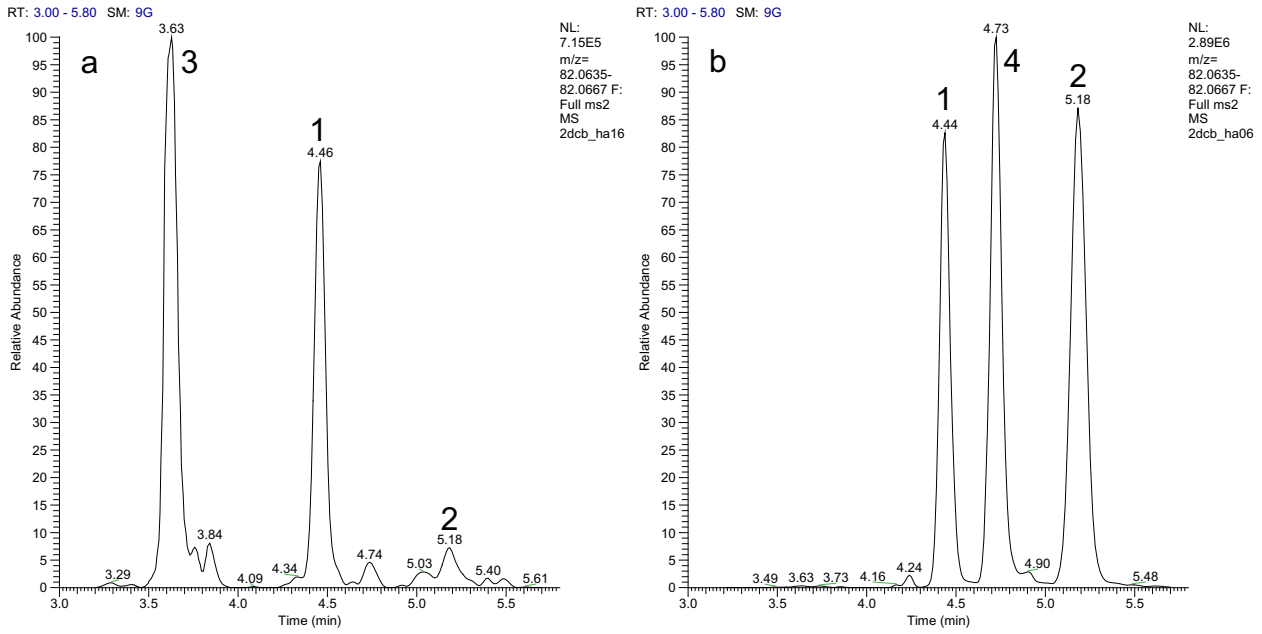

Fig. 2. HPLC–HRMS extracted ion current of m/z 82.0651 (tolerance 20 ppm) of sample extracts derivatised with hydroxylamine; Panel a: highly irradiated nutmeg material; Panel b: highly irradiated cashew nut material; 1 – 2-DCB oxime, 2 – 2-TCB oxime, 3 – 2-decylcyclobutanone oxime (tentative), 4 – 2-tetradecenylcyclobutanone oxime (tentative).

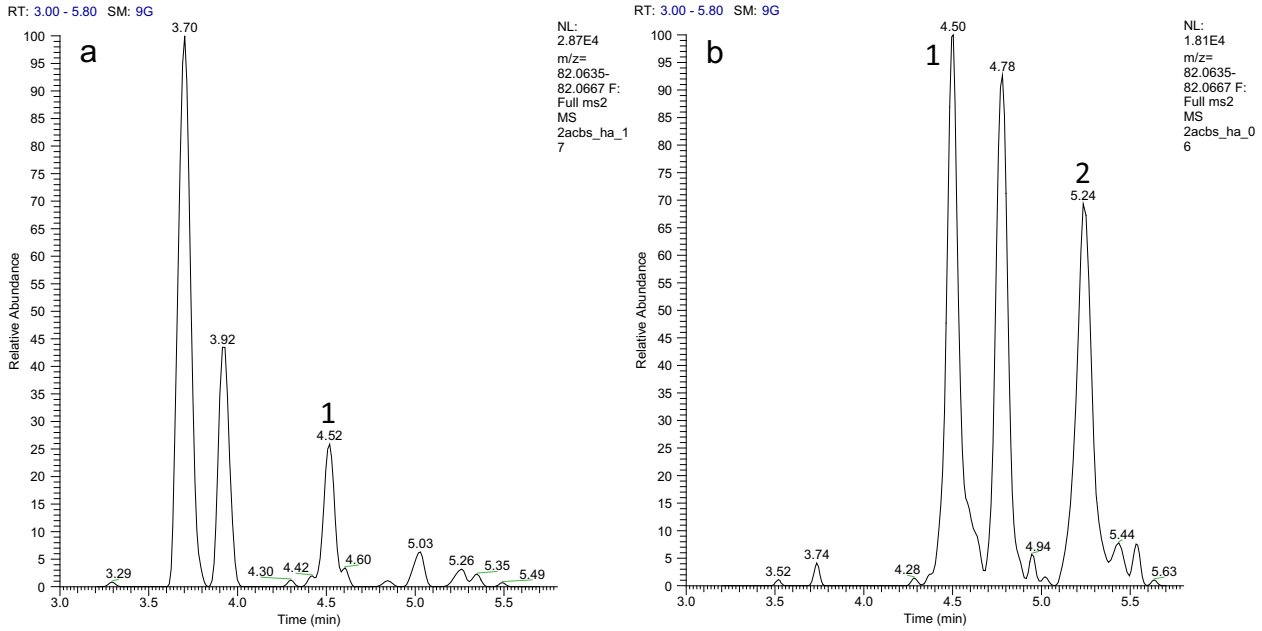

Fig. 3. HPLC–HRMS extracted ion current of m/z 82.0651 (tolerance 20 ppm); Panel a: nutmeg sample irradiated at 400 Gy; Panel b: cashew nut sample irradiated at 100 Gy; 1 – 2-DCB, 2 – 2-TCB.

free of 2-ACBs were spiked with 0, 5, 10, and 25 μL of a mixture containing 0.2 μg/mL of 2-DCB and 2-TCB. This corresponded to levels of 0, 2, 4, and 10 ng/g; the recovery experiment was repeated three-times on different days. Recovery was taken as the slope of the linear regression of the observed concentrations over the expected concentrations. The detection limit was determined according to ISO 11843:2000 (International Organization for Standardizaton, 2000). The arithmetically estimated detection limits were verified by spiking the appropriate amount in of 2-ACBs to cashew nut and nutmeg and submitting the spiked samples to the whole analytical work flow. Precision was not included in the validation programme as the confirmed presence of 2-ACBs alone would be taken as evidence that the concerned food was irradiated.

3.5. Applicability of the MSPD–HPLC–HRMS method for detecting irradiated cashew nut and nutmeg

The applicability of the MSPD method in combination with HPLC–HRMS for detection of food irradiation was tested by analyzing cashew nut and nutmeg samples that were treated with low doses of ionizing irradiation to estimate what level of irradiation could be detected. The same rule as given in EN 1785:2003 was

Table 2
Summary of method performance parameters of the tested method to detect irradiated food; n.d. = not determined.

Material	Analyte	Parameter	Method		
			GC–MS	GC–MS/MS	HPLC–HRMS
Cashew nut	2-DCB	LOD [μg/kg]	50	50	5
		Mean recovery [%]	103	103	101
	2-TCB	LOD [μg/kg]	50	50	8
		Mean recovery [%]	97	97	53
	Lowest detected irradiation dose		700	700	100
Nutmeg	2-DCB	LOD [μg/kg]	n.d.	n.d.	8
		Mean recovery [%]	n.d.	n.d.	102
	2-TDCB	LOD [μg/kg]	n.d.	n.d.	8
		Mean recovery [%]	n.d.	n.d.	87
	Lowest detected irradiation dose		>1000	>1000	400

applied to detect irradiation: presence of at least one of the target 2-ACB in an amount greater than the detection limit (signal to noise ratio larger than 3:1). 2-DCB as well as 2-TCB were detected in all irradiated cashew nut samples even in those irradiated with a dose as low as 100 Gy (Fig. 3, Panel b). For nutmeg, sensitivity was lower, but irradiation at 400 Gy was clearly detected (Fig. 3, Panel a). Modeling the lowest detectable dose of irradiation showed a value of 20 Gy for cashew nut and 140 Gy for nutmeg. These results convincingly demonstrate the superiority of this MSPD-LC–MS approach over the EN 1785:2003 for cashew nuts and nutmeg.

3.6. Survey of cashew nut and nutmeg

To verify that 2-ACBs do not occur in materials not labeled as irradiated, 26 cashew nut and 14 nutmeg samples were processed with MSPD, derivatised, and measured with HPLC–HRMS. Neither 2-decyl, 2-dodecyl, 2-tetradecenyl, nor 2-tetradecylcyclobutanone could be detected in any of the samples. In addition, several samples have also been measured with the less sensitive EN 1785:2003 for comparative purpose; none was positive for 2-ACBs.

4. Conclusions

The European Standard EN 1785:2003 allowed the detection of irradiated cashew nut at an irradiation dose of 700 Gy; however, for nutmeg neither 2-DCB nor 2-TCB could be detected at an irradiation dose of 1000 Gy. For the further improvement of the sensitivity to detect irradiation a novel method of analysis was developed, in-house validated and applied to several cashew nut and nutmeg samples. A combination of matrix solid phase dispersion, conversion to oxime derivatives, and measurement with HPLC–HRMS delivered detection limits of smaller than 10 μg/kg for 2-DCB and 2-TCB. Irradiation at doses of less than 100 Gy (cashew nuts) and 400 Gy (nutmeg) could be detected. Twenty-six cashew nut and 14 nutmeg samples obtained from retailers in 10 different EU Member States tested negative for 2-ACBs using the optimized HPLC–HRMS method. Consequently, there is no reason to believe that 2-ACBs are not unique indicators of treatment with ionizing radiation.

Conflict of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.01.032.

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