Verification of the applicability of EN 1785:2003 for the detection of irradiation treatment of cashew nuts and nutmeg

Administrative Arrangement
SANCO/2013/G4/SI2.663671
Final Report

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2015
Abstract
The applicability of the international standard method of analysis EN 1785:2003 “Detection of irradiated food containing fat - Gas chromatographic/mass spectrometric analysis of 2-alkylcyclobutanones.” for cashew nuts and nutmeg was investigated and verified for irradiation doses larger than 1kGy. A novel method, capable of detecting doses larger than 100 Gy, was developed and validated. 40 test samples from the EU market did not show any indication for irradiation.
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Executive summary

The irradiation of dried aromatic herbs, spices and vegetable seasonings is authorised at EU level by Directive 1999/3/EC at a maximum overall average absorbed radiation dose of 10 kGy. Irradiation of food products and ingredients must be indicated by proper labelling. For checking compliance with legislation a number of analytical methods have been elaborated and standardised by the European Committee for Standardization (CEN). Among this suite of methods is EN 1785:2003 Detection of irradiated food containing fat – Gas chromatographic/mass spectrometric analysis of 2-alkylcyclobutanones. The standard specifies a method for the identification of irradiation treatment of food containing fat.

In 2008 a study was published by Variyar et al, claiming the natural occurrence of 2-alkylcyclobutanones in cashew nut (Anacardium occidentale) and nutmeg (Myristica fragrans), thus disproving the hypothesis that 2-alkylcyclobutanones are radiolytic degradation products of fat, which can serve as unique markers for irradiation treatment of fatty foods. For nutmeg those findings have not been confirmed by another independent study, although the same analytical approach was applied.

Triggered by those conflicting reports the relevant CEN Technical Committee 275 Working Group 8 worries about the fact that the Variyar et al study is used by the international community not only to question the validity of EN 1785 but all the other methods for the detection of irradiated foods, which have been elaborated in Europe. Therefore, the Directorate–General Health and Food Safety has commissioned the Joint Research Centre (JRC) of the European Commission to repeat the study of Variyar et al in order to verify whether 2-alkylcyclobutanones are indeed unique markers for irradiated food and whether the provisions of EN 1785:2003 are still valid.

This study evaluated the appropriateness of EN 1785:2003 Foodstuffs – Detection of irradiated food containing fat – Gas chromatographic/ mass spectrometric analysis of 2-alkylcyclobutanones, for the detection of radiolysis products in cashew nuts and nutmeg, which led to the following conclusions:

a) The analytical method prescribed in EN 1785:2003 is suitable to detect irradiation of cashew nut samples at average absorbed doses of 1 kGray and above. This is fully in line with the provision of the standard and the validation data cited therein, which were obtained by an interlaboratory study including raw chicken, pork, liquid whole egg, salmon and Camembert.

b) For nutmeg the sensitivity of EN 1785:2003 for the detection of radiolysis products is lower, which might be caused by matrix interference. Nutmeg is not only a high fat product, but contains essential oils in addition, which might interfere.

c) An alternative method developed by JRC, which is based on matrix solid phase extraction and subsequent separation and detection of 2-alkylcyclobutanones by high performance – high resolution mass spectrometry, is more sensitive than EN 1785:2003. This method allowed to detection of 2-alkylcyclobutanones in cashew nuts irradiated at 100 Gray and in nutmeg irradiated at 400 Gray. This is an improvement in sensitivity by a factor of 5-10 compared to EN 1785:2003.

d) None of the 26 cashew nut and 14 nutmeg samples purchased in different EU Member States contained traces of 2-alkylcyclobutanones.
e) The results of the Variyar et al study were not confirmed in this survey.

f) In the light of the results of this study, it is unlikely that 2-acylcyclobutanones occur naturally in cashew nut and nutmeg.

g) Therefore, this study confirms the validity of the analytical method EN 1785:2003 for the detection of food irradiation through the analysis of 2-acylcyclobutanones in cashew nut and nutmeg.

h) Suitable alternatives to the method described in EN 1785:2003 exist, which are more sensitive, less laborious and require smaller amounts of chemicals. In case of revision of the standard, those alternative methods could be considered as candidates for replacement.
Introduction

Food irradiation is a non-thermal process for the inactivation of micro-organisms and pests (e.g. insects) to control spoilage of food and extend its shelf-life. Ionizing radiation, e.g. produced by a $^{60}\text{Co}$ source, an electron beam or X-ray generator, kills micro-organisms by breaking chemical bonds in molecules that are important for sustaining life (nucleic acids, proteins). This process is known as radiolysis. The radiation dose applied varies depending on the kind of food and the targeted species to be inactivated. The applied dose ranges from 300-500 Gray (Gy) for inactivation of pests and parasites up to 30 kGray (kGy) for sterilization of spices and seasonings. The interaction of ionizing radiation with the food matrix does not only kill harmful bacteria but also generates low amounts of radiolysis products, mostly from the fat contained in the food.

The safety of irradiated food has been thoroughly studied; in 1981 the Joint Expert Committee on Food Irradiation (JECFI), established by the United Nations World Health Organization/International Atomic Energy Agency/Food and Agriculture Organisation (WHO/IAEA/FAO), came to the overall conclusion that irradiation of food up to an overall average dose of 10 kGy presents no toxicological hazard and introduces no special nutritional or microbiological problems [1]. In 2011, the European Food Safety Authority (EFSA) delivered a scientific opinion on the chemical safety of irradiation of food taking into account newer information. EFSA concluded that there is not an immediate cause for concern related to the consumption of irradiated food [2].

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 [3]. The community list of foodstuffs which may be treated with ionizing radiation to the exclusion of all others and the maximum radiation doses authorised are given in Directive 1999/3/EC. The only harmonised 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 [4]. Proper labelling of irradiated food products and ingredients is required at EU level as well as by the FAO/WHO Codex Alimentarius [5]. For checking compliance with legislation a number of analytical methods have been elaborated and standardised by the European Committee for Standardization (CEN). Among this suite of methods is EN 1785:2003 Detection of irradiated food containing fat - Gas chromatographic/mass spectrometric analysis of 2-alkylcyclobutanones. 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 (2ACBs) after gas chromatographic (GC) separation. The method has been successfully tested in interlaboratory 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 2ACBs have never been detected in non-irradiated samples of this product.

In 2008, a study was published by Variyar et al [6], claiming the natural occurrence of 2ACBs in cashew nut ($\text{Anacardium occidentale}$) and nutmeg ($\text{Myristica fragrans}$), thus disproving the hypothesis that 2ACBs are radiolytic degradation products of fat, which can serve as unique markers for irradiation treatment of fatty foods. The authors claimed 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 2ACBs in the mentioned food products. For nutmeg those findings have not been confirmed by another independent study, although the same analytical approach was applied [7]. Meanwhile, other reports came out supporting the fact that 2ACBs do not occur naturally [8, 9]. The latter reports did not use EN 1785 for the determination of 2ACBs, but claimed that their analytical methodology is superior or at least equivalent.

Triggered by those conflicting reports the relevant CEN Technical Committee 275 Working Group 8 worries about the fact that the Variyar et al [6] study is used by the international community not only to question the validity of EN 1785 but all the other methods for the detection of irradiated foods, which have been elaborated in Europe. Likewise, EFSA in their Scientific Opinion on the Chemical Safety of Irradiation of Food [2] noted that "...as no further evidence of the natural occurrence of 2ACBs has yet been reported, it would be pertinent to treat these findings with some caution until the results are validated by further experimental work".

Terms of Reference (as agreed in the Administrative Arrangement)

The primary aim of the study was to clarify whether 2ACBs occur as natural constituents in non-irradiated cashew nut and nutmeg. Therefore, the Directorate-General for Health and Food Safety (DG SANTÉ) has asked the Joint Research Centre, Institute for Reference Materials (JRC-IRMM) to repeat the study of Variyar et al in order to verify whether 2ACBs are markers for irradiated food and whether the provisions of EN 1785:2003 are valid.

Specifically, it was agreed that the JRC will:

Task 1: Procure non-irradiated cashew nut and nutmeg samples from the EU market (obtained from retail outlets and producers of snacks and spices). As irradiation is not widely applied in the EU the likelihood of obtaining non-irradiated products on the market is high. However, spices may be irradiated and spice producers will be contacted to obtain authentic material.

Task 2: Several lots of non-irradiated samples will be irradiated in a research facility at various radiation doses.

Task 3: Implement EN 1785:2003 in JRC-IRMM and verify method performance according to the standard using irradiated and non-irradiated cashew nut and nutmeg samples (Task 1 and 2).

Task 4: Check whether 2ACBs are present in a suitable number of non-irradiated cashew nut and nutmeg samples (Task 1 and 2) using EN 1785:2003.

Task 5: Develop more exhaustive extraction conditions as compared to the one used in EN 1785:2003 for 2ACBs based on pressurized liquid extraction (PLE), ultrasound assisted extraction, Folch extraction and subject the obtained extracts to highly selective separation and detection methods such as gas-chromatography coupled to tandem mass spectrometry, comprehensive gas chromatography coupled to high-resolution time-of-flight mass spectrometry.
Task 6: Apply the most exhaustive extraction technique together with the most selective separation/detection approach to verify whether 2ACBs are naturally occurring substances in non-irradiated samples (Task 1)

**Experimental**

**Cashew nut and nutmeg samples**

Samples (pre-packaged) were obtained from retail outlets in a number of EU Member States. None of the samples were labelled as being irradiated. An overview of the origin of samples is given in Table 1. 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.

<table>
<thead>
<tr>
<th>Country of purchase</th>
<th>Cashew Number of samples</th>
<th>Nutmeg Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>BE</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>CZ</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>DE</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>ES</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>HR</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>HU</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>IT</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>RO</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>UK</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>26</strong></td>
<td><strong>14</strong></td>
</tr>
</tbody>
</table>

*Table 1: Sources of cashew nut and nutmeg samples*

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

Seven of the nutmeg samples were already ground and not treated any further. 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 ground nutmeg.

**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 for each material. 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, DE (details see Annex A).

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; for details see Annex A) at a different facility specialised in food irradiation (SynergyHealth, Etten-Leur, NL). Those highly irradiated samples were used to verify that EN 1785:2003 and all the developed alternative methods indeed are able to detect the 2-ACBs.

**Reference solutions**

2-dodecylcyclobutanone (2dDCB) and 2-tetradecylcyclobutanone (2tDCB) were purchased from Sigma-Aldrich (Belgium). They 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 2dDCB and 1 mg/mL 2tDCB in n-hexane. From these two stock solutions mixed working solutions of different concentrations were prepared.

**Stable-isotope labelled internal standard**

To facilitate the control of extraction, clean-up, and measurement a stable-isotope labelled analogue of 2-dodecylcyclobutanone was synthesised by the JRC.

Under very strong alkaline conditions the three hydrogens in the positions 2,4,4 of 2-dodecyl cyclobutanone were exchanged against deuterium to give 2,4,4-2H₃-2-dodecyl cyclobutanone (D₃-2dDCB). In brief, 53 mg of 2dDCB were dissolved in a mix of 1 mL aceton-d₆ (C₃₂H₆O) and 10 mL Methanol-d₁ (MeOD, CH₃O²H). 610 µL of 14 mol/L sodium deuterium oxide (NaO²H) in heavy water (²H₂O) 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 (¹HCl) in heavy water (²H₂O) 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 sulphate (Na₂SO₄). After filtration of the organic phase it was evaporated to dryness under vacuum.

The reaction product was cleaned up by preparative reversed-phase high-performance liquid chromatography. A Shimadzu LC20 AD solvent delivery system with a low pressure gradient unit delivered a flow of 4 mL/min of methanol (MeOH)/ 2-propanol (i-PrOH)/ water (H₂O) (63/27/10, v/v/v) to a Discovery HS C18 250x10 mm, 5µm, column (Supelco, 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 D₃-2dDCB in single MS high resolution mode. The fraction of the remaining flow containing D₃-2dDCB was collected. This fraction was concentrated and injected onto a Discovery HS F5 250x10 mm, 5µm, column (Supelco, USA) for additional clean-up under the same conditions as above.

From the final cleaned up product a solution of D₃-2dDCB equivalent to 200 µg 2dDCB per mL of n-hexane was prepared and used throughout the whole study.
Methods

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

**Figure 1** Overview of applied measurement techniques for the determination of 2ACBs in cashew nut and nutmeg samples (HPLC-HRMS, high-performance liquid chromatography – high resolution mass spectrometry).

**EN 1785:2003 Foodstuffs - Detection of irradiated food containing fat – Gas chromatographic/mass spectrometric analysis of 2-alkylcyclobutanones**

The European Standard EN 1785:2003 “Foodstuffs – Detection of irradiated food containing fat – Gas chromatographic/ mass spectrometric analysis of 2-alkylcyclobutanones” was applied to 23 samples (Cashew 15, Nutmeg 8; see also Table 8) with the following minor modifications: the internal standard 2-cyclohexylcyclohexanone was replaced by D₃-2-dodecylcyclobutanone 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, 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.

In brief, 5 g of test material were mixed with 5 g of sodium sulphate in a cotton extraction thimble. This was then extracted with n-hexane for six hours using an automated Soxhlet extractor (Büchi B-
The extract was dried in vacuum at 40 °C with a Laborota 4001 rotary evaporator (Heidolph, Germany), and 2 g of the extracted lipid were diluted to 10 mL with n-hexane after addition of 1 µg of D3-2-dDCB. Florisil (PR grade 60-100 mesh, Supelco, USA) was heated to 550 °C overnight; after cooling 20 parts of water were added to 100 parts of adsorbent (m/m), and left overnight to deactivate. Approximately 30 g of deactivated Florisil were poured into a glass chromatography column (20 x 300 m) containing n-hexane and allowed to settle; then 1 mL of the diluted extract (0.2 g/mL) was applied. Unbound compounds were washed off with 80 mL of n-hexane. The 2-alkylcyclobutanones were then eluted with 80 mL of n-hexane/ diethyl ether (99/1, v/v). The eluate was collected and taken to dryness under vacuum at 40 °C after addition of 200 ng each of methyl pentadecanoate and methyl heptadecanoate. The dry extract was reconstituted with 200 µL isooctane and 2 µL injected into the GC-MS.

The GC-MS consisted of an Agilent 6890 gas chromatograph connected to an Agilent 5973 inert mass spectrometer. Separation was afforded with a DB-5MS column (28m, 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 2dDCB and 2tDCB are easily accessible EN 1785:2003 is focusing on and validated for those two only.

**Alternative extraction methods**

To compare yields of different extraction approaches a cashew nut sample, blank of 2ACBs, 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.

For PLE the test portion was mixed with an equal amount of sodium sulphate 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 [11]: extraction temperature 100 °C, extraction pressure 1500 psi, preheating 5 min, static extraction 5 min, solvent flush 10 mL, nitrogen purge 60 s.

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

**GPC clean-up**
The extracts of the different extraction approaches were loaded onto a GPC column (BioRad BioBeads SX-3 33 cm length x 2.5cm I.D.) using a Valco Cheminert six-port, two-position low pressure valve (VICI Int, Switzerland) with a 5 mL sample loop and eluted with cyclohexane/ethyl acetate (50/50, v/v) at a flow rate of 5 mL/min, which was delivered by a Shimadzu LC-20 AD pump. 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 (Heidolph, Germany).

Matrix solid phase dispersion (MSPD)

For MSPD 0.5 g of a test material 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 x 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., UK).

To determine apparent recoveries and limit of detection 0.5 g of cashew nut and nutmeg free of 2ACBs were spiked with 0, 5, 10, and 25 µL of a mixture containing 0.2 µg/mL of 2dDCB and 2tDCB per millilitre. This corresponded to levels of 0, 2, 4, and 10 ng/g; the recovery experiment was done three-times (three different days).

Derivatisation of 2ACBs

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 millilitre of ACN/H2O (50/50, v/v). After a minimum of 60 min at room temperature the mixtures containing the 2ACB-oximes were inject without any further processing.

Detection of 2-acylcyclobutanones 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" MS. The MS was operated in positive chemical ionization mode with methane as reactant gas. Separation was afforded by a DB-1 column (15m, 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 Helium. 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. The following transitions were acquired in selected reaction monitoring (Table 2):

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precursor ion [Da]</th>
<th>Product ion [Da]</th>
<th>Dwell time [s]</th>
<th>Collision energy [eV]</th>
<th>Delay [s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2dDCB</td>
<td>239.2</td>
<td>95.1</td>
<td>0.05</td>
<td>7</td>
<td>0.02</td>
</tr>
<tr>
<td>2dDCB</td>
<td>239.2</td>
<td>109.1</td>
<td>0.05</td>
<td>7</td>
<td>0.02</td>
</tr>
<tr>
<td>D3-2dDCB</td>
<td>242.2</td>
<td>98.1</td>
<td>0.05</td>
<td>7</td>
<td>0.02</td>
</tr>
<tr>
<td>D3-2dDCB</td>
<td>242.2</td>
<td>112.1</td>
<td>0.05</td>
<td>7</td>
<td>0.02</td>
</tr>
<tr>
<td>Methyl pentadecanoate</td>
<td>257.2</td>
<td>103.1</td>
<td>0.03</td>
<td>13</td>
<td>0.02</td>
</tr>
<tr>
<td>Methyl pentadecanoate</td>
<td>257.2</td>
<td>117.1</td>
<td>0.03</td>
<td>13</td>
<td>0.01</td>
</tr>
</tbody>
</table>
Detection of 2-acylcyclobutanones 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 (all Shimadzu, Benelux), and an Orbitrap Elite mass spectrometer (Thermo Scientific, Belgium). Chromatographic separation was afforded by an Ascentis express Phenyl column (100x2.1 mm, particle size 2.6 µm; Supelco, USA) with mobile phase A: H₂O/ formic acid (999/1,v/v) and mobile phase B: ACN/ 2-propanol/ 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, 15min 45% B with a flow rate of 300 µL/min.

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 15000. This was followed by a product ion scan with Higher-Energy Collisional Dissociation (HCD) fragmentation at resolving power 15000, normalized collision energy 60, and isolation width 5 amu. The following precursor masses were selected during the indicated time window (Table 3):

<table>
<thead>
<tr>
<th>Precursor m/z</th>
<th>Start time</th>
<th>Stop time</th>
</tr>
</thead>
<tbody>
<tr>
<td>226.2165</td>
<td>3.45</td>
<td>3.85</td>
</tr>
<tr>
<td>255.7500</td>
<td>4.25</td>
<td>4.65</td>
</tr>
<tr>
<td>281.2635</td>
<td>4.65</td>
<td>5.70</td>
</tr>
</tbody>
</table>

Table 3: Precursor mass list

The presence of a common product ion with m/z 82.0651 (C₅H₈N; for d₃-2dDCB m/z 85.0840, C₅H₅₋₃H₃N), which fully preserves the 2-substituted cyclobutanone structure, was seen as specific indicator for the presence of 2ACBs. The full scan data was used to confirm molecular mass and elemental composition.

Results and Discussion

Verification of the performance of EN 1785:2003

According to EN 1785:2003, samples are considered to be irradiated when:

a) at least one of the two 2ACBs targeted has been positively identified, and
b) the estimated concentration exceeds the concentration yielding a signal to noise ratio of 3 to 1 (detection limit) in the least sensitive ion.

The accreditation standard for testing and calibration laboratories, ISO/IEC 17025:2005 [10], requires laboratories to verify the performance of standardized testing methods when implemented in a laboratory. In keeping with this principle, experiments were made to estimate recovery (as a proxy for trueness) and detection limit for cashew nut samples spiked with 2dDCB and 2tDCB (see Table 4, and Figure 2).

<table>
<thead>
<tr>
<th>Spike level [µg/kg]</th>
<th>Recovery 2dDCB [%]</th>
<th>Recovery 2tDCB [%]</th>
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</thead>
<tbody>
<tr>
<td>100</td>
<td>106</td>
<td>107</td>
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<tr>
<td>200</td>
<td>99</td>
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<tr>
<td>2000</td>
<td>105</td>
<td>92</td>
</tr>
<tr>
<td>Detection limit</td>
<td>50 µg/kg</td>
<td>50 µg/kg</td>
</tr>
</tbody>
</table>

Table 4 Recovery and detection limit for 2ACBs in cashew nut

Figure 2 GC-MS extracted ion currents of m/z 84.1, 98.1, 112.1 (tolerance 1 amu) of a cashew nut sample spiked at 100 µg/kg (EN 1785).
<table>
<thead>
<tr>
<th>Irradiation dose (Gy)</th>
<th>Detected in Cashew</th>
<th>Detected in Nutmeg</th>
</tr>
</thead>
<tbody>
<tr>
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<td>No</td>
</tr>
<tr>
<td>400</td>
<td>No</td>
<td>No</td>
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<tr>
<td>1000</td>
<td>Yes(^a)</td>
<td>No</td>
</tr>
</tbody>
</table>

*Table 5* Sensitivity of EN 1785:2003 to detect irradiation of cashew nut and nutmeg based on the markers 2dDCB or 2tDCB; *a* – detection of 2dDCB

The applicability of the method was tested by analysing cashew nut and nutmeg samples that were treated with low doses of ionizing irradiation to estimate what level of irradiation could be detected by the method. The outcome of these experiments is summarized in Table 5. Figure 3 and Figure 4 show chromatograms for cashew nut and nutmeg. Based on these findings it can be stated that EN 1785:2003 is applicable for the detection of low-level irradiation doses in cashew nuts between 400 and 700 Gy. The detection limits for the radiolysis products 2dDCB and 2tDCB were 50 µg/kg for both. The average crude fat content as determined by Soxhlet was 46 g per 100 g cashew nut, so the detection limit in relation to crude fat is about 100 µg/kg.

EN 1785:2003 was not able to detect irradiation at low doses (< 1kGy) in nutmeg. One reason for this deficiency might be the fact that nutmeg fat (‘nutmeg butter’, whose main component is trinmyristin a triglyceride with three myristic acid moieties) 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 top of the Florisil column, which might have negatively impacted the clean-up efficiency. This becomes also obvious when comparing the cashew nut chromatogram (Figure 3) to the nutmeg chromatogram (Figure 4) where the background signal is much higher.
Figure 3 GC-MS extracted ion currents of m/z 84.1, 98.1, 112.1 (tolerance 1 amu) of a cashew nut sample irradiated at 700 Gy.

Figure 4 GC-MS extracted ion currents of m/z 84.1, 98.1, 112.1 (tolerance 1 amu) of a nutmeg sample irradiated at 1000 Gy.
Another reason for this deficiency could be that neither 2dDCB nor 2tDCB, the two markers targeted by EN 1785:2003, were the most abundant markers of irradiation in nutmeg. The main fatty acid of 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 2ACBs, the prominent peak eluting around 8.6 min in Figure 4 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).

**Alternative methods for the detection of 2ACBs in cashew nut and nutmeg**

Since the development of the analytical method that is described in EN 1785:2003 several improvements in instrumental measurement techniques were introduced in laboratories.

**Detection of 2ACBs 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. The ionization mode had to be changed from electron ionization, as prescribed in EN 1785:2003, to chemical ionization (with methane as reactant gas) to create meaningful precursor ions for further fragmentation and selected reaction monitoring. Unfortunately, in direct comparison this approach did not lead to significant improvements of the method's sensitivity to detect irradiation (see chromatogram in Figure 5). The main reason for this was possibly the fact that the ionization efficiency in chemical ionization is poorer than in electron ionization, which offset the benefits of the improved selectivity of GC-MS/MS. Therefore, this technique was not further investigated.

![Figure 5](image.png)

**Figure 5** Extracted ion chromatograms of the GC-MS/MS measurement of a cashew nut sample spiked at 100 µg/kg with 2dDCB and 2tDCB; top: transition 267->95 for 2tDCB (11.34 min), middle: transition 242->98 for D3-2dDCB (10.13 min), bottom: transition 239->95 for 2dDCB (10.15 min)
Detection of 2ACBs by high performance liquid chromatography – high-resolution mass spectrometry (HPLC-HRMS)

High performance liquid chromatography (HPLC) is a separation technique for which, opposite to GC, the analytes do not have to be volatile. Therefore, HPLC is predominantly used for more polar or very large molecules. But it can also be used for less polar molecules for which normally GC would be the technique of choice. 2ACBs lend themselves well to GC but there are a few reasons why HPLC is a good choice for their separation: larger amounts of injection solution, equivalent to more sample, can be loaded, which is beneficial for lowering the detection limit, and the tolerance for lipids is higher which relaxes the requirements for the sample clean-up.

For 2ACBs 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 2ACBs. In HPLC-MS electro spray ionization (ESI) is the most commonly used source of ions. This is not very efficient for native 2ACBs but the ionization efficiency of 2ACBs can be significantly improved through a very simple derivatisation reaction leading to the formation of the respective oximes (Figure 6); then very specific signals can be recorded (see product ion spectrum in Figure 7). Because of those reasons HPLC-MS offers a great potential for faster methods of analysis with improved limits of detection for 2ACBs.

![Figure 6](image-url)  
**Figure 6** Structure of 2ACB oxime; R=C\textsubscript{10}H\textsubscript{21} 2-decyl cyclobutanone oxime; R=C\textsubscript{12}H\textsubscript{25} 2-dodecyl cyclobutanone oxime; R=C\textsubscript{14}H\textsubscript{29} 2-tetradecyl cyclobutanone oxime

![Figure 7](image-url)  
**Figure 7** Product ion spectrum of 2tDCB oxime (the spectra of 2DCB and 2dDCB are identical)
Because of the limitations of the GC-MS and -MS/MS methods and a recent publication of Leung et al. [8], who claimed that HPLC-MS has superior sensitivity for the detection of 2ACBs, that approach was further used in combination with alternative extraction and clean-up procedures for the sensitive detection of those radiolysis products.

**Tentative identification of 2-decylcyclobutanone (2DCB) and 2-tetradecenyl cyclobutanone (2tDenCB)**

It is to be expected that the profile of 2ACBs formed by irradiation somehow reflects the fatty acid profile of the irradiated food. The dominant fatty acid in nutmeg is myristic acid of which the corresponding irradiation marker would be 2-decylcyclobutanone. In cashew nuts oleic acid, a mono-unsaturated fatty acid, is very dominant and its irradiation product would be 2-tetradecenyl cyclobutanone. Since no commercial sources for 2DCB or 2tDenCB reference material could be found, the occurrence of 2DCB and/or 2tDenCB was tentatively identified by elemental composition, product ion spectrum, and retention of the oxime in reversed-phase liquid chromatography (RP-LC). To that end highly irradiated cashew nut and nutmeg materials were prepared with MSPD and derivatised with hydroxylamine to obtain the oximes of the 2ACBs.

The elemental composition of 2DCB 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 2DCB oxime was 3.61 min. Since 2DCB oxime is a homologue of 2dDCB oxime (4.47 min) and 2tDCB oxime (5.20 min) the retention time differences between these peaks were expected to be very similar. The difference between 2dDCB oxime and 2tDCB oxime was determined to be 0.73 min. The retention time difference between the peak tentatively identified as 2DCB oxime and 2dDCB oxime was 0.86 min. Because of the excellent agreement of the calculated masses with the observed m/z of 2DCB oxime and its fragments in the product ion spectrum and the consistency with the predicted retention time, the peak at 3.61 min was tentatively identified as 2DCB oxime (see Figure 8).

![Figure 8](image-url)  
*Figure 8* Extracted ion current of m/z 82.0651 (tolerance 10 ppm) of a highly irradiated nutmeg material after derivatisation with hydroxylamine. The prominent peak at 4.47 min
is 2-dodecylcyclobutanone oxime, the minor peak at 5.20 min is 2-tetradecylcyclobutanone oxime, and the largest peak at 3.61 min is tentatively 2-decylcyclobutanone oxime.

The elemental composition of 2tDenCB oxime is C_{18}H_{33}NO (m/z 280.2635 for [M+H]^+) with the same typical product ions of 2ACB oximes. Because of the presence of a mono-unsaturation a retention time shorter than the corresponding fully saturated 2-tetradecylcyclobutanone but longer than 2-dodecylcyclobutanone was expected.

The following ions were measured for the peak with a retention time of 4.76 min: m/z 280.2636, 82.0648, 96.0806, and 110.0963. Therefore, the peak at 4.76 min was tentatively identified as 2-tetradecenylcyclobutanone (see Figure 9).

**Figure 9** Extracted ion current of m/z 82.0651 (tolerance 10 ppm) of a highly irradiated cashew nut material after derivatisation with hydroxylamine. The peak at 4.46 min is 2-dodecylcyclobutanone oxime, the peak at 5.21 min is 2-tetradecylcyclobutanone oxime, and the largest peak at 4.76 min is tentatively identified as 2-tetradecenylcyclobutanone oxime.

**Extraction of 2ACBs**

Variyar et al [6] claimed that a combination of supercritical fluid extraction (SFE) using carbon dioxide at 80 °C and a pressure of 146-148 bar in combination with thin-layer chromatographic (TLC) clean-up of the extract allowed them to detect and identify 2ACBs in cashew nut and nutmeg, while the method described in EN1785:2003 failed to do so.

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

Supercritical carbon dioxide at 80 °C and a pressure of 146-148 bar has a density of 410.4 kg/m³, which allows a satisfactory extraction of hydrophobic substances such as triglycerides from plant matrices. Since 2ACBs are less hydrophobic than triglycerides, extraction solvents that are more
polar than \( n \)-hexane and supercritical carbon dioxide should be able to remove 2ACBs with higher selectivity from the sample matrix.

Pressurized solvent extraction (PLE), which employs elevated pressure and temperature during extraction to improve extraction yields, was applied to selected samples with two different polar extraction solvents, ethyl acetate (EtOAc) [11] and acetonitrile (ACN). Another approach is UAE. Here the test material is mixed with an extraction solvent and then sonicated with ultrasound for a period of time.

When analysing solid samples the most common approach is to transfer the analyte from the solid phase into the liquid phase by extraction with an appropriate solvent system. Since this almost always co-extracts unwanted compounds a clean-up of the extract is advisable or even essential. The European Standard EN 1785:2003 follows this scheme. 2ACBs are extracted with the majority of all lipids in the test material into the liquid phase which is then cleaned up with Florisil column chromatography to remove the unwanted lipids. It is well known that the capacity of Florisil to retain lipids from crude extracts is limited; therefore, an alternative clean-up method was sought.

Gel permeation chromatography (GPC) is a widely used approach to separate small organic analytes from large amounts of lipids with high efficiency. The separation principle is based on the size of molecules, with larger molecules travelling faster than smaller ones. 2ACBs are less than 1/3 the size of the predominant triglycerides in edible fat. GPC can be automated, columns can be used many times, and loading capacities are up to one gram of lipid, which is approximately five times as much as with the Florisil column described in EN 1785:2003. About 98% of the lipids can be eliminated with little loss of the smaller analyte.

An altogether different approach to solid sample analysis is matrix solid phase dispersion (MSPD) [12]. Developed in 1992 it consist of thoroughly mixing an amount of the solid sample (matrix) with an amount of a coarse particulate material (solid phase) like sand, silica, modified silica, etc. Through the forces acting during the mixing process cell structures are disrupted and the matrix is evenly dispersed over the surface of the solid phase. This makes analytes easily accessible for solvents. The matrix solid phase dispersion is then filled into an empty polyethylene syringe barrel and compressed. Finally, the compounds of interest can be eluted with appropriate solvents. Through proper choice of the solid phase and the elution solvent high extraction yields with little co-extraction can be obtained. The benefits of MSPD are significant savings in time and solvent usage. A disadvantage is the practical limitation to the test portion size.

Pressurized liquid extraction (PLE) allowed the extraction to be performed within 15 min and ultrasound assisted extraction (UAE) within 30 min as opposed to the 6 h of the Soxhlet extraction of EN 1785:2005. 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). Figure 10 shows the extraction yields for the different
extraction approaches for cashew nut. It is evident that none of the approaches outperformed the others in terms of extraction yield.

The same equivalence of extraction methods was observed for nutmeg (Figure 11), but a different pattern of 2ACBs was observed. In irradiated cashew nut, which contains substantial amounts of palmitic and stearic acid, there appeared to be slightly more 2tDCB than 2dDCB at altogether high levels, while in irradiated Nutmeg, which is rich in myristic acid, very little 2tDCB and only slightly more 2dDCB were found. The major marker was 2-decylcyclobutanone (2DCB) which was found at 10-times the level of 2dDCB.

Figure 10 Extraction yields for 2-dDCB and 2-tDCB in cashew nut with four different extraction systems; top row: Soxhlet (SOX), second row: Ultrasound assisted extraction (UAE), third row: Pressurized liquid extraction with acetonitrile (PLE-ACN), fourth row: Pressurized liquid extraction with ethyl acetate (PLE-EtOAc); left column: 2-dodecyl cyclobutanone, right column: 2-tetradecyl cyclobutanone; solid line: regression fit
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 matrix solid phase dispersion [11] was investigated. Cashew nut and nutmeg samples spiked at 0, 2, 4, and 10 µg/kg were prepared on three different days and measured. 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 [13].

Table 6 lists the performance characteristics in terms of recoveries and detection limits for cashew nut and nutmeg. For verification of the detection limits see chromatograms in Figure 12 to Figure 15. It is evident that the signal-to-noise ratios of the detected peaks are larger than 3, which is usually considered as the limit of detection.
Table 6 Performance characteristics of the MSPD-HPLC-HRMS approach for cashew nut and nutmeg for 2dDCB and 2tDCB

Figure 12 HPLC-HRMS extracted ion current of m/z 82.0651 (tolerance 20 ppm) for a cashew nut sample spiked with 4 µg/kg 2dDCB

Figure 13 HPLC-HRMS extracted ion current of m/z 82.0651 (tolerance 20 ppm) for a cashew nut sample spiked with 10 µg/kg 2tDCB

Figure 14 HPLC-HRMS extracted ion current of m/z 82.0651 (tolerance 20 ppm) for a nutmeg sample spiked with 10 µg/kg 2tDCB
Figure 15 HPLC-HRMS extracted ion current of m/z 82.0651 (tolerance 20 ppm) for a nutmeg sample spiked with 10 µg/kg 2tDCB.

The applicability of MSPD was tested by analysing cashew nut and nutmeg samples that were treated with low doses of ionizing irradiation to estimate what level of irradiation could be detected by the method. The same rule as given in EN 1785:2003 was applied to detect irradiation: presence of at least one of the target 2ACBS in an amount greater than the detection limit (signal to noise ratio larger than 3:1). The outcome of these experiments is summarized in Table 7 (chromatograms for cashew nut and nutmeg in Figure 16 and Figure 17).

<table>
<thead>
<tr>
<th>Irradiation dose (Gy)</th>
<th>Detected in Cashew</th>
<th>Detected in Nutmeg</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
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<td>1000</td>
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</table>

Table 7 Sensitivity of the matrix solid dispersion method for detection of irradiation in cashew nut and nutmeg based on the markers 2dDCB or 2tDCB.

Even though recovery for 2tDCB appears to be inferior to the EN 1785:2003 approach, the detection limits have improved by more than a factor of 5. This is certainly owed to the fact that HPLC-HRMS is highly selective and that more than twice as much test material could be injected compared to GC-MS. Processing time from comminuted test material to eluted extract was below 10 min, total solvent consumption below 10 mL of ACN, and irradiation was detected at even the lowest dose (100 Gy) in cashew nut. For nutmeg sensitivity was lower, but irradiation at 400 Gy was clearly detected. Modelling 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.
Survey of the natural occurrence of 2ACBs in cashew nut and nutmeg

The profile of 2ACBs in foods subjected to ionizing irradiation is dependent on the fatty acid profile of that particular food but possibly also on the way extraction, clean-up and detection are performed. Therefore, not a single specified marker should be used for identification but the occurrence of any 2ACB during the analysis.
To verify that no 2ACBs could be detected in materials not labelled as irradiated, all 26 cashew nut and 14 nutmeg samples were processed with MSPD, derivatised, and measured with HPLC-HRMS. In none of these samples any 2-decyl, 2-dodecyl, 2-tetradecenyl, and 2-tetradecyl cyclobutanones could be detected. In addition several samples have also been measured with the less sensitive EN 1785 for comparative purpose. The individual results are listed in Table 8.

<table>
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<th>Country of Purchase</th>
<th>Lot</th>
<th>Material</th>
<th>Results EN 1785: 2003</th>
<th>Results MSPD-HPLC-HRMS</th>
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<td>HU</td>
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<td>L312694</td>
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<td>Frutos secos</td>
<td>Frit Ravich</td>
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</table>

The decision whether a sample has been irradiated is not based on the amount of fat derived radiolysis products, but only on their presence/absence (zero-tolerance principle), which is governed by the sensitivity of the method. For this reason EN 1785:2003 has not been applied to all samples, since the developed method combining matrix solid phase dispersion, derivatisation and detection by HPLC-HRMS was shown to be much more sensitive.
Conclusions

In order to verify whether 2ACBs occur naturally in cashew nut and nutmeg and to check whether the EN 1785:2003 ‘Foodstuffs – Detection of irradiated food containing fat – Gas chromatographic/mass spectrometric analysis of 2-alkylcyclobutanones’ is applicable to detect irradiation of cashew nut and nutmeg, an investigation was performed with 26 cashew nut and 14 nutmeg samples obtained from retailers in 10 different EU member states. Two of the cashew nut and two of the nutmeg samples were exposed to γ-rays at doses of 100, 400, 700, and 1000 Gy. For the EN 1785:2003 method detection limits of 50 µg/kg (109 µg/kg crude fat) were determined and for cashew nut an irradiation dose of 700 Gy could be detected based on the presence of 2-dodecylcyclobutanone. For nutmeg even at the highest irradiation dose of 1000 Gy neither 2-dodecyl nor 2-tetradecylcyclobutanone could be detected.

For the further improvement of the sensitivity to detect irradiation, a new 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-dodecyl and 2-tetradecylcyclobutanone; irradiation at doses of less than 100 Gy (cashew nuts) and 400 Gy (nutmeg) could be detected. Table 9 summarizes these performance characteristics.

Based on the results obtained in this study, it can be concluded that EN 1785:2003 is capable of detecting irradiation doses > 1kGy in cashew nuts and possibly also in nutmeg. None of the 40 tested samples showed any indication of the presence of 2ACBs above 10 µg/kg. Consequently, there is no reason to believe that 2ACBs are not unique indicators of treatment with ionizing radiation.

<table>
<thead>
<tr>
<th>Material</th>
<th>Analyte</th>
<th>Parameter</th>
<th>Method</th>
<th>GC-MS</th>
<th>GC-MS/MS</th>
<th>HPLC-HRMS</th>
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<td>2dDCB</td>
<td>LOD [µg/kg]</td>
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<td>50</td>
<td>5</td>
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<td></td>
<td></td>
<td>Mean recovery [%]</td>
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<td>103</td>
<td>101</td>
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<tr>
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<td>2tDCB</td>
<td>LOD [µg/kg]</td>
<td>50</td>
<td>50</td>
<td>8</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Mean recovery [%]</td>
<td>97</td>
<td>97</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lowest detected irradiation dose</td>
<td>700</td>
<td>700</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Nutmeg</td>
<td>2dDCB</td>
<td>LOD [µg/kg]</td>
<td>n.d.</td>
<td>n.d.</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mean recovery [%]</td>
<td>n.d.</td>
<td>n.d.</td>
<td>102</td>
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<tr>
<td></td>
<td>2tDCB</td>
<td>LOD [µg/kg]</td>
<td>n.d.</td>
<td>n.d.</td>
<td>8</td>
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<tr>
<td></td>
<td></td>
<td>Mean recovery [%]</td>
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<td>n.d.</td>
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<td>Lowest detected irradiation dose</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>400</td>
<td></td>
</tr>
</tbody>
</table>

Table 9 Summary of method performances; n.d.=not determined

References

4. List of Member States’ authorisations of food and food ingredients which may be treated with ionising radiation. Official Journal of the European Union C 283/5.


ANNEX A

Irradiation certificate:

**JRC.GAD.GEEL**

**HZB Helmholtz Zentrum Berlin**

**Dr. Ing. Helke**

**E PT**

** технический**

**стейк**

**Tel:** +49 30 261 6205

**Fax:** +49 30 261 64029

**info@hzbschicht.de**

**Dr. Zinner**

**Rectifier**

**Berlin, 11143**

---

**Strahlungsquelle:** Co 60 Gamma

**Probenart:** Lebensmittelproben

**Probenbezeichnung:**

10 Gy/Masse 1, 40 Gy/Masse 1, 80 Gy/Masse 1

70 Gy/Masse 1, 100 Gy/Masse 1, 170 Gy/Masse 1

**Messort:** PTW Unidos 3 mit Ionisationskammer Typ 21019

**Mittlerer Dosisleistung:** 2,69 Gy/h, 18,1 Gy/h, 26,0 Gy/h

**Bestrahlungszeit:** 38,73 h

**Gesamtdosiswerte:**

10 Gy, 40 Gy, 70 Gy, 100 Gy

**Bemerkungen:** Die einkreuzte aufgestellten Probenwürfel wurden zur Gewährleistung einer möglichst homogenen Bestrahlung nach der Hälfte der Bestrahlungszeit einmal um 180° gedreht. Zur Berücksichtigung von Ablösungen an der Fläche wurden die nominellen Bestrahlungszeiten um 5% erhöht.

---

**Forschung Zentrum Berlin**

**Materialien und Energie**

**PTW Unidos 3, Therapie (PT)**

**Dr. Zinner**

**Berlin, 11143**
Certificate of Gamma Irradiation

Synergy Health Ede B.V., Soevereinstraat 2, 4879 NN Etten-Leur, the Netherlands,
Telephone: +31 (0)76 5041055, certifies the following irradiation treatment
in accordance with :

*    licence number KEW 2012/0286-05,
    issued by the Dutch Ministry of Economic Affairs, Agriculture and Innovation.
*    the national and international Quality Assurance System Standards:
    ISO 9001:2008 and additionally for CE marked medical devices:

The irradiation dose applied is controlled by Synergy Health Ede B.V.
The calibration of the dosimetry is carried out by the National
Physical Laboratory in the United Kingdom.

Product description: Rest
Customer reference: ERM-BD286/ERM-BD286
Irradiation facility: GS6000 Pallet irradiator
Irradiation date: 01-12-2013
Irradiation dose: 5,0 kGy Min. 15,0 kGy Max.
Order number: 26145399

Synergy Health Etten Leur QA 02-12-2013
Synergy Health

Orderline number 2614539901
Customer I.R.M.M.
Product description Rest
Remarks
Customer reference ERM-BD285/ERM-BD2

Measurement Results

<table>
<thead>
<tr>
<th>PIN code</th>
<th>No.</th>
<th>Position</th>
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<tr>
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<td>1</td>
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<tr>
<td>1</td>
<td>2</td>
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</tr>
<tr>
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<td>1</td>
<td>MAX REF.</td>
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<tr>
<td>4</td>
<td>2</td>
<td>MIN REF.</td>
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</table>

Operator Ronald Goosen

Irradiation unit GS6000
Irradiation date 1-12-2013 / 1-12-2013
Density 0.40 kg/dm³
Requested dose 5.0 kGy Min.
15.0 kGy Max.

Dose Min. 8.6 kGy
Dose Max. 10.9 kGy

Plant Manager/Quality Department
A great deal of additional information on the European Union is available on the Internet. It can be accessed through the Europa server http://europa.eu.

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Working in close cooperation with policy Directorates-General, the JRC addresses key societal challenges while stimulating innovation through developing new methods, tools and standards, and sharing its know-how with the Member States, the scientific community and international partners.

Serving society
Stimulating innovation
Supporting legislation

doi: 10.2787/901421