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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.