EN 1785:2003 method for the identification of ionizing treatment in poultry meat products: optimization of the accelerated solvent extraction procedure

Maria Campaniello, Rosalia Zianni, Andrea Chiappinelli, Annalisa Mentana, Valeria Nardelli

National Reference Laboratory for the Treatment of Foods and their Ingredients with Ionizing Radiation, Chemistry Department, Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, Foggia, Italy

Abstract

X-ray irradiation is a non-thermal technology used for food preservation and sanitization to prolong shelf life. To evaluate the fraudulent use of X-ray treatment, in this study, an accelerated solvent extraction method was optimized for the extraction of lipids and 2-dodecylcyclobutanone (2-DCB), reducing analysis time and solvent consumption compared to Soxhlet extraction reported in the standard EN 1785:2003 method. The qualitative confirmation method was tested on different processed poultry meats, *i.e.*, sausages and hamburgers, irradiated at different dose levels, that is, 0.5, 1.0, and 3.0 kGy. The analytical parameters investigated, namely method linearity, selectivity, minimum dose level, diagnostic sensitivity and specificity, and recovery, showed that the proposed method is suitable for routine analysis in official controls to determine 2-DCB as an irradiation marker.

Correspondence: Rosalia Zianni, National Reference Laboratory for the treatment of foods and their ingredients with ionizing radiation, Chemistry Department, Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, via Manfredonia 20, 71121 Foggia, Italy.
E-mail: rosalia.zianni@izspb.it

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Introduction

To enhance food safety and prolong shelf life, new thermal and non-thermal processing technologies are emerging as promising alternatives to traditional methods (Campaniello *et al*., 2020). Among these, ionizing radiation treatments (such as γ-ray, e-beam, X-ray) have gathered increasing industrial attention as effective cold sterilization methods (Zhang *et al*., 2023). Irradiation can inhibit pathogens and spoilage by disrupting the genetic material of microorganisms (DNA and RNA), thereby impeding their reproduction and the onset of diseases or food spoilage (Chiesa *et al*., 2022). Irradiation does not make foods radioactive, does not substantially increase internal temperature, and does not cause nutritional reduction and/or alteration, and so the treated foodstuffs are safe to consume (Rigante *et al*., 2024). The European regulatory framework for food irradiation, comprising a list of food and food ingredients authorized for treatment and the relative dose levels (European Commission, 1999a and 1999b). The gray (Gy) is the unit used to quantify absorbed radiation, with 1 Gy equal to 1 joule of absorbed energy per kilogram of material, and in food irradiation, doses in the range of kilograys (kGy) are typically employed (Tomaiuolo *et al*., 2023; Palermo *et al*., 2024). The World Health Organization (1999) suggests that the recommended dose for irradiation should not surpass 10 kGy. Although the great potential of this technology is well-recognized, the consumption of irradiated foods is a subject of controversy primarily because most consumers, who are uninformed, intimidated, and influenced by terminology, fear health risks (Mangiacotti *et al*., 2013). It is the European Union (EU) legal requirement that treated foods or foods that contain an irradiated ingredient be labelled correctly as such, reporting also the facilities of treatment, to allow informed choice (European Commission, 1999a and 1999b). Hence, official checks of irradiated food on the market are a requirement of EU regulations to distinguish between irradiated food and not-irradiated (NI) food, to regulate international trade, and to meet labelling requirements. The European Committee for Standardization (CEN) has

developed, validated, and standardized several analytical detection methods (*Supplementary Table 1*) based on radiation-induced physical, chemical, or biological changes in foods (Mangiacotti *et al*., 2013; Marozzi *et al*., 2013; Campagna *et al*., 2014; Marrone *et al*., 2014). Among them, the standard chemical confirmatory method EN 1785:2003 is based on the determination of 2-alkylcyclobutanones (2-ACBs), in particular 2-dodecylcyclobutanone (2-DCB) (European Committee for Standardization, 2003). ACBs are radiolytic products in irradiated lipid-based foods, formed *via* acyl-oxygen bond cleavage in triglycerides during irradiation, also generating other volatile compounds (Bliznyuk *et al*., 2022; Zianni *et al*., 2023). Specifically, 2-DCB originates from triglycerides with palmitic acid content (Campaniello *et al*., 2020; Zianni *et al*., 2022). To date, 2-ACBs have not been detected in foods treated with other technological treatments (Jeong *et al*., 2014); however, advanced methodologies are needed to confirm their exclusive presence in irradiated foods. The determination of 2-DCB in the standard method EN 1785:200 validated by CEN is based on Soxhlet lipid extraction using hexane for 6-8 hours, sample clean-up by column chromatography purification, and detection by gas chromatography with mass spectrometric detection (GC-MS). This method is hard-working for routine analysis due to long and solvent-consuming extraction and a complex clean-up step. Different analytical methods with improvement of extraction, clean-up, and analysis have recently been developed for 2-DCB (Soncin *et al*., 2012; Lacivita *et al*., 2019; Campaniello *et al*., 2020). In particular, considering the lipid extraction phase, accelerated solvent extraction (ASE) could represent an alternative to Soxhlet extraction, as an automated, rapid, and more environmentally friendly procedure (Obana *et al*., 2005; Mentana *et al.*, 2022). ASE uses solvents at high pressures and temperatures above the boiling point; thus, the solvents solubilize the targeted compounds and penetrate the sample matrices better than at atmospheric conditions and room temperature. Until now, ASE applications have been widespread to lipophilic organic contaminants such as polychlorinated biphenyls, dioxins, furans, and pesticide residue analyses (Nardelli *et al*., 2020). In recent decades, there has been an increasing consumption of poultry meat and related processed products, linked to the advantageous ratio between nutritional value and price. The use of physical treatments, such as irradiation, is effective against the growth of *Salmonella*, *Campylobacter*, and *Listeria*, which are considered the main pathogens present in the poultry supply chain (Campaniello *et al.,* 2020). EU legislation allows the irradiation of poultry meat up to 7 kGy. In this context, poultry is considered a suitable matrix for this research work. To date, several works have been published in the last two years regarding the quality of chicken meat subjected to ionizing radiation (Hashim *et al.,* 2024), but poor literature regarding methods of analysis for 2-DCB (Obana *et al.,* 2005; Li *et al.,* 2017).

This study aims to develop and optimize an ASE method for the rapid extraction of the total lipids and 2-DCB in poultry meat products, to replace the traditional solvent extraction of the standard procedure EN 1785:2003. To evaluate the suitability of the method on processed and irradiated meat, the following parameters were considered namely analytical linearity, selectivity, minimum dose level (MDL), diagnostic sensitivity and specificity, and recovery. Finally, this study could be proposed for application to food safety control plans of several irradiated lipid-containing foodstuffs.

Materials and Methods

Chemicals and reagents

The standards of the 2-DCB (purity ≥95%) and 2-cyclohexylcyclohexanone (purity ≥95%), the latter used as internal standard (IS), were purchased from Sigma-Aldrich (Buchs, Switzerland). n-hexane analytical grade, cyclohexane analytical grade, isooctane for pesticide residue analysis, and diethyl ether analytical grade were purchased from Honeywell Fluka™ (Seelze, Germany). Extrelut® NT and Florisil gel (150-250 µm) were acquired from Merck Life Science S.r.l. (Darmstadt, Germany). Stock standard solutions of 2-DCB and IS at 1000 µg/mL were prepared in isooctane and stored at -20°C. Working standard solutions at 10 and 0.5 µg/mL of 2-DCB and at 10 and 0.1 µg/mL of IS were prepared in n-hexane. Solvent-matched calibration (SMC) curve was prepared at 5, 10, 20, 40, 80, and 120 ng/mL 2-DCB concentrations in n-hexane with IS (final concentration of IS 10 ng/mL) by appropriate dilution of working standard solutions. The point at concentration at 5 ng/mL of 2-DCB with IS at 10 ng/mL was employed as quality control (QC) to assess the system suitability, stability, and repeatability of the data acquisition process. Each point of the SMC was analyzed in triplicate to obtain a mean straight line, and the standard deviation of the measurements was calculated and reported as error bars (*Supplementary Table 2* and *Supplementary Figure 1*).

X-ray irradiation treatment

All samples were purchased from local markets. Processed meat products, namely chicken sausages (CS), turkey hamburger (TH), and turkey/chicken sausages (TCS), were used in this work. In this work, NI and irradiated homogenized samples were used. Irradiation was carried out in plastic bags, using an X-ray irradiator (RS-2400, Radsource Inc., Suwanee, GA, USA). The list of analyzed matrices were reported in Table 1, specifically: 9 NI samples (3 for each matrix); 12 irradiated samples at 0.12 and 0.5 kGy (2 samples for CS, TH, TCS at two dose levels), 18 irradiated samples at 0.5, 1.0 and 3.0 kGy (2 samples for CS, TH, TCS at three dose levels); 2 NI samples of TH spiked before extraction at 40 ng/g to recovery calculation.

Table 1. List of analyzed samples for validation procedure: chicken sausages, turkey hamburger, turkey/chicken sausages. Specifically, for all matrices, 3 not-irradiated samples, 2 irradiated samples at 0.12 and 0.5 kGy (2 for each dose), 3 irradiated samples at 0.5, 1.0, and 3.0 kGy (2 for each dose), 2 not-irradiated samples spiked at 40 ng/g were analyzed.

Matrix	CS	TH	TCS	Total
Not-irradiated samples	3	3	3	9
MDL (kGy)[a]	4	4	4	12
Linearity (kGy)[b]	6	6	6	18
Recovery (%)[c]	–	2	–	2

CS, chicken sausages; TH, turkey hamburger; TCS, turkey/chicken sausages; MDL, minimum dose level. [a]0.12-0.5 kGy; [b]0.5-1.0-3.0 kGy; [c]40 ng/g.

Accelerated solvent extraction procedure

Thermo Scientific™ Dionex™ ASE™ 350 ASE system (Thermo Fisher Scientific, Waltham, MA, USA) was used for fat extraction employing nitrogen as carrier gas. The 33 mL volume extraction cells in stainless steel were fitted with a cellulose filter and a stainless-steel frit. Afterwards, 10 g of the homogenized sample was mixed with 10 g of Extrelut® NT used as a dispersion agent, and to reduce the solvent volume. Lipid extracts were evaporated to dryness, to constant weight, at 40°C under nitrogen flow using an automated solvent evaporation system TurboVap® II (Biotage AB, Uppsala, Sweden).

In order to optimize ASE conditions, three variables were evaluated, *i.e.*, solvent type, number of static cycles, and % flush volume. Cyclohexane and n-hexane, both suitable for lipid extraction, were tested as extracting solvents. In ASE extraction, the static cycles represent the number of static automated extractions. This lengthy exposure to solvents and consequent matrix swelling improved the solvent penetration into the sample interstices and their contact with the analytes (Yu *et al*., 2010). During the entire extraction procedure, the amount of solvent employed remains constant, and it is divided into the established static cycles. Flush volume or flushing is the fresh solvent amount added between each cycle as a percentage (mL/mL) of the extraction cell volume, in our case of 33 mL. Table 2 reports the ASE operating conditions employed for 4 different extraction programs (Programs 1, 2, 3, and 4) used to optimize extraction parameters, evaluating the gravimetric yield of lipid content.

Column chromatography purification procedure

A chromatography column with deactivated Florisil® was used for the clean-up procedure of ASE dried-lipid extracts, following the protocol reported in the standard method EN 1785:2003.

Gas chromatography with mass spectrometric detection analysis

In this work, extracted and purified samples were analyzed by a gas chromatograph equipped with a programmed temperature vaporization injector coupled to a mass triple quadrupole TSQ Quantum MS (Thermo-Fisher Scientific, Waltham, MA, USA). Sample injection was carried out by TriPlus autosampler (Thermo-Fisher Scientific, Waltham, MA, USA). The GC-MS method was previously developed by Campaniello *et al*. (2020), and its operating conditions are briefly reported below. A DB5-MS capillary column (Agilent Technologies, Santa Clara, CA, USA) of 30 m and 0.25 mm i.d. with a 0.25 µm stationary phase (5% diphenyl- and 95% dimethylpolysiloxane) was used to perform separation. The oven GC conditions were set as follows: 50°C (held 4 min); 230°C (rate 15°C/min, held 1 min) and 310°C (rate 16°C/min, held 1 min). Helium (99.9995% purity) (Sapio S.r.l., Monza, Italy) was used as a carrier gas with a constant flow of 1.0 mL/min. The mass spectrometry conditions were set as follows: electron ionization, an ion voltage of 70 eV, and an ion source temperature of 250°C. The selected ion monitoring mode was used for data acquisition, monitoring two predominant ions, 98 and 112 m/z for 2-DCB and 98 and 180 for IS.

Assessment of 2-dodecylcyclobutanone

The presence of 2-DCB, as a chemical marker of irradiation treatment, was determined following the criteria reported in the standard method EN ISO 1785:2003 and in our previous works (Campaniello *et al*., 2020; Zianni *et al*., 2022). Briefly, the sample was classified as irradiated if all three of the three following conditions are true: i) chromatographic peak at a retention time comparable (±0.5%) with the 2-DCB retention time of QC is present; ii) signal-to-noise ratios of this peak extracted at m/z 98 and 112 that were greater than 3; iii) percentage ratio (RA %) values of the peak areas at m/z 98 and 112 (RA % = $R_{A112}/_{A98}$ *100) within the range 13-35%.

Evaluation of the method's analytical performance

The analytical performances of the qualitative confirmatory ASE/GC-MS method were evaluated in terms of instrument linear-

Table 2. Operating conditions of accelerated solvent extraction and gas chromatography with mass spectrometric detection analysis.

ASE conditions (cell size 33 mL)	Program 1	Program 2	Program 3	Program 4
Solvent	n-hexane	n-hexane	n-hexane	Cyclohexane
Temperature (°C)	50	50	50	50
Static cycles	2	3	2	2
Static time (min)	5	5	5	5
Flush (%)	60	60	100	100
Purge (s)	180	180	180	180
Lipid recovery (%)				
Mean ± CV%	81.0±5.2	74.5±0.9	84.5±7.5	94.0±1.5

ASE, accelerated solvent extraction; CV, coefficient of variation percentage.

Table 3. 2-dodecylcyclobutanone matrix concentration in irradiated and spiked turkey hamburger samples.

Samples	Matrix concentration (ng/g)	Recovery % (R)	Matrix concentration* · R (ng/g)
Spiked samples (40 ng/g) (mean n=2)	27	67.5	–
Irradiated samples (mean n=2)	0.5 kGy	2	– 3
	1 kGy	7	– 11
	3 kGy	20	– 30

kGy, kilograys.

ity, method linearity, selectivity, MDL, recovery percentage, diagnostic sensitivity, and specificity. EURACHEM 2014 guidelines were applied to evaluate methods and to show if it is fit for purpose, but suggested analytical performances were integrated (EURACHEM, 2014). Linearity is an important property of methods used to make measurements at a specific range of concentrations. The linearity of this analytical procedure for 2-DCB was evaluated within the concentration range of SMC to verify the direct proportionality of instrumental response. Moreover, to consider the matrix effect on method linearity, the matrix curve (MC) at the irradiation doses of 0.5, 1.0, and 3.0 kGy was determined. Selectivity of this analytical method, that is, its ability to measure accurately 2-DCB in the presence of interferences in the sample matrix, was checked by examining NI samples. MDL is related to the sensitivity and is the minimum dose of irradiation that can be evaluated by means of 2-DCB identification. For the evaluation of MDL, a total of 12 samples were prepared and specifically: 2 samples for each matrix at a dose of 0.12 kGy and 2 samples for each matrix at a dose of 0.5 kGy. Diagnostic sensitivity was defined as the fraction of true positive samples (irradiated and NI samples) that were identified among the total number of positive samples. Diagnostic specificity was the fraction of true negative samples (irradiated and NI samples) that were identified among the total number of negative samples. For the evaluation of diagnostic sensitivity and specificity, the same 9 NI and 18 irradiated samples were used for the verification of selectivity and the calculation of the MC, respectively. The accuracy was defined as the fraction of correctly classified samples of the total number of samples. Finally, recovery was calculated on TH samples spiked with 2-DCB at a concentration of 40 ng/g in order to ensure that the ASE/GC-MS method was accurate.

Results and Discussion

Accelerated solvent extraction optimization

Optimization of the ASE method was carried out in duplicate using the TH sample (labeled fat content of 7.1%) following the four programs reported in Table 2. The results were evaluated based on lipid gravimetric recovery. The extraction temperature was set at 50°C, a mild condition to avoid possible sample degradation, and the static time was adjusted to 5 minutes to minimize the total extraction time without compromising the final yield. The selection of an appropriate extraction solvent, especially in terms of polarity, is one of the major challenges in developing an ASE method (Mentana *et al*., 2022).

The selection of a suitable extraction solvent was performed by testing the efficiency of extraction of n-hexane, the solvent used in EN 1785:2003, and cyclohexane. These two solvents are structurally different but are similar in terms of lipophilicity. Moreover, both solvents, having high *Log P* values, are suitable for the extraction of 2-DCB, which is also a lipophilic and apolar compound. Program 4, namely cyclohexane as solvent, 2 static cycles, and 100% of flush volume, providing a gravimetric yield of lipid greater than 90%, was the best, and for this reason, it was used as the extraction procedure. The raw ASE extracts of NI, irradiated, and spiked samples were purified as described in the "Column chromatography purification procedure" section and then analyzed by means of GC-MS.

Analytical performances of accelerated solvent extraction/Gas chromatography with mass spectrometric detection method

The linearity of this analytical procedure for 2-DCB was evaluated by SMC and MC, and both curves showed a good linearity with a correlation coefficient ≥0.999. Matrix effect was evaluated by means of proportionality between irradiation dose and 2-DCB peak area. MDL was evaluated on low-dose irradiated meat samples, and 0.5 kGy was found to be suitable for identifying the treatment for all matrices. Regarding diagnostic sensitivity and specificity, the proposed method was accurate with 100% of correctly identified samples, at the MDL and beyond, for each matrix studied. Moreover, as shown in the extracted ion chromatograms (Figure 1), the employed method was suitable for performing the separation and identification of 2-DCB from other matrix peaks. Indeed, in all NI samples, no peaks identifiable as 2-DCB were observed (dotted line), while 2-DCB was present in all irradiated samples. Finally, % recovery was calculated on TH samples spiked with 2-DCB at a concentration of 40 ng/g as reported in Table 3. Moreover, Table 3 showed the final concentration of 2-DCB in irradiated samples at different doses, calculated according to EN 1785:2003, using the following equations (Eqs. 1-3). The relative response of 2-DCB was calculated in relation to IS, that is *F*, as reported in Eq. 1:

$$F = \frac{A_{cy}}{A_{IS} \cdot \rho_{cy}} \quad \text{[Eq. 1]}$$

where A_{cy} was the peak area of 2-DCB at 98 m/z; A_{IS} was the peak area of IS at 98 m/z; and ρ_{cy} was the mass concentration of 2-DCB (µg/mL). Then, the average of all *F* responses was calculated to get F_{av} and to calculate the mass concentration of 2-DCB in the sample ($\rho_{cy/s}$) expressed as µg/200 µL, with Eq. 2:

$$\rho_{cy/s} = \frac{A_{cy/s}}{A_{IS/s} \cdot F_{av} \cdot 5} \quad \text{[Eq. 2]}$$

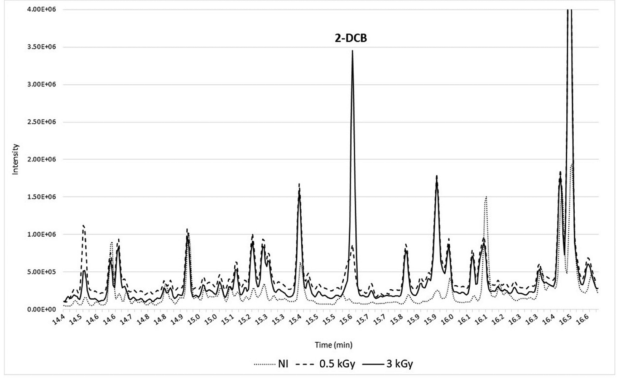

Figure 1. Extracted ion chromatograms derived from the sum of m/z 98 and 112, obtained by accelerated solvent extraction/gas chromatography with mass spectrometric analyses of turkey hamburger samples: not-irradiated (NI) (dotted line), irradiated at 0.5 kilograys (kGy) (dashed line), and 3.0 kGy (solid line). 2-DCB, 2-dodecylcyclobutanone.

where $A_{cy/s}$ was the peak area of 2-DCB at 98 m/z in the sample; $A_{IS/s}$ was the peak area of IS at 98 m/z in the sample; and F_{av} was the average of all ratios as calculated using Equation 1.

Moreover, the mass fraction of 2-DCB (w_{cy}), considering the correction for lipid content (µg/g of fat), had to be calculated, following Eq. 3:

$$w_{cy} = \frac{\rho_{cy/s}}{m_0} x\ 1000 \qquad \text{[Eq. 3]}$$

where $\rho_{cy/s}$ was the 2-DCB mass concentration in the sample µg/200 µL, as calculated using Eq. 2; m_0 was the weight of the lipid taken for the Florisil® expressed in mg. The calculated average recovery, equal to 67.5% (Table 3), resulted similar to what was reported by Obana *et al.* (2005), using the ASE procedure and ethyl acetate as solvent to extract 2-DCB from chicken thighs. At last, comparing the analyzed samples, no differences were found in terms of the area of the 98 m/z ion of 2-DCB. This is probably due to a similar fat content, and in particular of palmitic acid, for the various analyzed matrices.

Conclusions

The EN 1785:2003 method allows the use of different methods for lipid extraction, suggesting some of them (*e.g.*, Soxhlet). In this work, an ASE method, a rapid and automated extraction, was proposed as an alternative for lipid extraction and 2-DCB determination in irradiated poultry meat products. ASE conditions were optimized in terms of solvent type, number of static cycles, and % of flush volume. Among these factors, cyclohexane as solvent, 2 as the number of static cycles, and 100% as the flush volume, were found to be the best conditions in terms of lipid extraction. The ASE procedure resulted in being suited for increasing extraction efficiency, reducing time, and being a more environmentally friendly procedure. Furthermore, the proposed ASE method, together with GC-MS, was applied to several processed and irradiated poultry products, *i.e.*, chicken, turkey, and mixed, to identify 2-DCB as an irradiation marker. For all matrices, MDL was 0.5 kGy, which is applicable to samples irradiated at doses at least equal to those reported in the standard method and compliant with the commercial dose used for these matrices. The method resulted in being selective and specific without false positives or false negatives starting from MDL. Moreover, the average recovery of 67.5%, in spiked samples is analogous to other reported studies. In conclusion, the ASE/GC-MS procedure proposed in this paper allows the correct identification of 2-DCB and has proven its suitability for routine analysis in official controls as an alternative to the currently available standard method. Because of these important outcomes, this method could be extended to other animal and vegetal matrices to determine 2-DCB and also other 2-ACBs as irradiation markers.

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Online supplementary material:
Supplementary Table 1. List of the European standard procedures for the detection of irradiated food.
Supplementary Table 2. Solvent-matched calibration curve.
Supplementary Figure 1. Mean solvent-matched calibration curve.