NUKLEONIKA 2009;54(2):115–121

ORIGINAL PAPER

A study on optimization of sample preparation for gas chromatographic methods used in routine irradiated foodstuffs detection*

Marian Virgolici,
Andrei V. Medvedovici,
Mihaela M. Manea,
Mihalis Cutrubinis,
Ioan V. Moise,
Rodica Georgescu

Abstract. The study is focused on the optimization and minimization of sample preparation procedures given in EN 1784 and EN 1785 standardized methods for the detection of irradiated foodstuffs which contain fats. The techniques used in this study were gas chromatography (GC) with a flame ionization detector (FID) for hydrocarbons (HC) detection and GC coupled with mass spectrometry (MS) for 2-alkylcyclobutanones detection. Our work revealed that alternative extraction and clean-up procedures as well as alternative injection modes like large volume injection can significantly reduce the total analysis time and costs with comparable results.

Key words: irradiated foodstuffs detection • gas chromatography

M. Virgolici✉, M. M. Manea, M. Cutrubinis, I. V. Moise, R. Georgescu
Department of Multipurpose Irradiation Facility (IRASM),
Horia Hulubei National Institute of Physics and Nuclear Engineering (IFIN HH),
407 Atomistilor Str., Com. Magurele, Ilfov County, 077125, P. O. Box MG-6, Romania,
Tel.: +0040 21 4042320, Fax: +0040 21 4575331,
E-mail: mvirgolici@nipne.ro,
virgolicimarian@yahoo.com

A. V. Medvedovici
Department of Analytical Chemistry,
Faculty of Chemistry,
University of Bucharest,
90/92 Panduri Str., 050663, Bucharest, Romania

Received: 5 November 2008
Accepted: 12 January 2009

Introduction

Detection of irradiated foodstuffs, which contain fats, performed using gas chromatographic standardized detection methods of hydrocarbons [3] and 2-alkylcyclobutanones [4] is, generally, of low productivity and a time and resource consuming process. Large volumes of high purity solvents are used for exhaustive extraction procedures of fats and separation of hydrocarbons (HC) and 2-alkylcyclobutanones (2-ACB) from the interfering matrix. Also, the corresponding large volumes of organic solvent wastes are produced through concentration of lipid and targeted radiolysis product extracts.

A solution for this problem could be alternative lipid extraction procedures from the literature [8] which allow a reasonable sample processing time, less expensive solvents, reagents and waste treatment costs.

Sometimes also the fat content in the tested food samples can reach extremely low concentrations (< 1%) which increases the specimen mass and solvent volumes for efficient lipid extraction.

The simplest solution in this case could be the development of more sensitive chromatographic methods which can compensate for the lower concentrations of the target radiolysis products.

Other obstacles which may occur can be related with the unknown exact fat composition of the tested samples, especially for foods for which interlaboratory

* Presented on the International Conference on Recent Developments and Applications of Nuclear Technologies, 15-17 September 2008, Białowieża, Poland.

data is unavailable in the standardized methods or even for mixed foodstuffs.

This obstacle can also be overcome by using or developing a fatty acid profiling chromatographic method for identification and fatty acid composition determination.

In the present article, a study was undertaken to optimize two standardized gas chromatographic methods [3, 4], considering extraction procedures, chromatographic method development for lower limits of detection and the use of MIDI, Inc.'s method (Sherlock Microbial Identification System MIS) [6, 7] combined with GC-MS for fatty acid profiling and structural confirmation. Also, the ESR method for detection of free radicals formed in meat bones and crustaceous shells [1] was applied for intercomparison purposes and confirmation of absorbed irradiation doses taken by the meat samples used in the present study.

Materials and methods

Meat and bone samples of chicken, salmon, crab and shrimp were obtained from the Romanian market. Samples were stored before and after irradiation in a freezer at –20°C.

Irradiation of the studied samples was performed in a SVST Co-60 gamma irradiator at Multipurpose Irradiation Facility IRASM (IFIN HH). Absorbed doses used in the present study were 0, 2, 7 and 10 kGy, measured using the oscilloscopic method with ethanol--chlor-benzene (ECB) dosimeters (relative standard deviation of ± 2%) [5].

Fatty acids composition was performed using the Sherlock 6.0 Microbial Identification System (MIDI, Inc. USA) [6] on a GC 6890N (Agilent Technologies) gas chromatograph (GC) equipped with an autosampler, a classical split/splitless injector (S/SL), an ultra 2 non polar capillary column (i.d. 0.32 mm; length 25 m; stationary phase phenyl, methyl-polysiloxane) and a classical flame ionization detector (FID).

Chromatographic method used was Sherlock's RTSBA6 consisting in a hot (250°C) split injection (50:1) of 2 μl of fatty acid methyl ester (FAME) extracts, followed by separation on a capillary column in a 28 K/min temperature gradient from 170°C to 288°C and non-specific detection with FID. Hydrogen was used as carrier gas in constant flow mode. FAME identification was made by retention time in RTSBA6 naming table (~ 300 FAME) comprised of a time window of 3.1 min defined by FAME C9:0 and C20:0. The naming table was calibrated in retention time and response factors with a calibration mixture of FAME (MIDI, Inc.'s rapid and sensitive calibration mixture 1300AA) with concentrations of 0.01, 0.005 and 0.0025%.

Sample preparation consisted of four steps: saponification, methylation (derivatization), liquid-liquid extraction and cleanup using MIS reagents for the RTSBA6 method. Initial samples consisted of 100 μl of methanolic lipid solution (~ 10 mg/ml) prepared from pure lipids obtained by solvent extraction procedures [3, 4].

Data analysis was performed automatically by MIS [7], and then only FAME with abundance (%) higher than 1% were considered.

For structural confirmation of retention time identified FAME, the secondary channel of GC6890N was used with a mass selective detector (MSD) in its configuration: GC injector Gerstel Cooled Injection System (CIS4) Programmable Temperature Vaporizer (PTV), capillary column HP5-ms (length 30 m, i.d. 0.25 mm, stationary phase phenyl, methyl-polysiloxane) and Agilent 5975 EI inert MSD equipped with electron ionization source and hyperbolic quadrupolar mass analyzer for positive ions with m/z ranging from 2 to 1050 amu. The chromatographic method consisted in a hot (250°C) split injection of 1 μl of the FAME extract (same sample prepared for MIS) followed by the separation on capillary column in a temperature gradient of 20 K/min from 170°C to 288°C in constant flow mode at 1.3 ml/min. MSD parameters were: transfer line temperature 280°C, electron ionization (EI) source with electrons of energy 70 eV, EI source temperature 230°C, quadrupole temperature 180°C, lenses and electron multiplier voltage (EMV) on detector (high energy dinode HED) were set through standard tune (scan from 2–700 amu) with PFTBA at above parameters.

Data analysis was made using Agilent Enhanced Chemstation software (version D.02.00.275), Automated Mass Spectral Deconvolution and Identification System AMDIS (version 2.62). Mass spectral assignament and compound identification was made using the experimental spectra for reference FAME from a MIS calibration mixture and reference spectra from NIST 2005 library (~ 200 thousand compounds).

Main hydrocarbons (HC $C_{n-1:0}$ and $C_{n-2:1}$) and 2-alkylcyclobutanones formed as radiolysis products in salmon, crab and shrimp meat were predicted from identified fatty acids.

Standardized [3, 4] and optimized hydrocarbon and 2-alkylcyclobutanones detection methods were performed on the GC-MS channel described above.

The EN 1784 classic optimized chromatographic method consisted in a hot (250°C) splitless (1 min) injection of 1 μl (HC extract with internal standard 1–4 ng/μl in n-hexane) followed by capillary column separation (HP5-ms column, constant flow at 1 ml/min, carrier gas helium) in two-step temperature gradient 10 K/min from 50°C to 130°C and 5 K/min from 130°C to 230°C. MSD parameters were: transfer line hold constant at 280°C, EI source at 230°C and quadrupole at 180°C, acquisition mode SCAN from 50 to 300 amu [3].

Increased sensitivity of HC chromatographic method consisted in the optimization of injection parameters as follows: PTV CIS4 used in solvent vent mode, cold (10°C) injection of 5 μl (HC extract with internal standard 0.1–4 ng/μl in n-hexane), followed by solvent vent optimized at 4 ml/min carrier gas (helium) and 0.0 psi for 1 min, splitless injection at 300°C for 1.5 min and injector cleanup at 350°C for 2 min. Capillary column, column flow, temperature program and MSD parameters were the same as for the classic method for comparison purposes. Injection temperature (Fig. 1) and solvent vent flow were optimized for minimization the loss of analites during concentration in the PTV injector.

The EN 1785 classic optimized chromatographic method consisted in a hot (250°C) splitless (1 min) injection of 1 μl (2-ACB extract with internal standard 0.25–2 ng/μl in n-hexane) followed by capillary

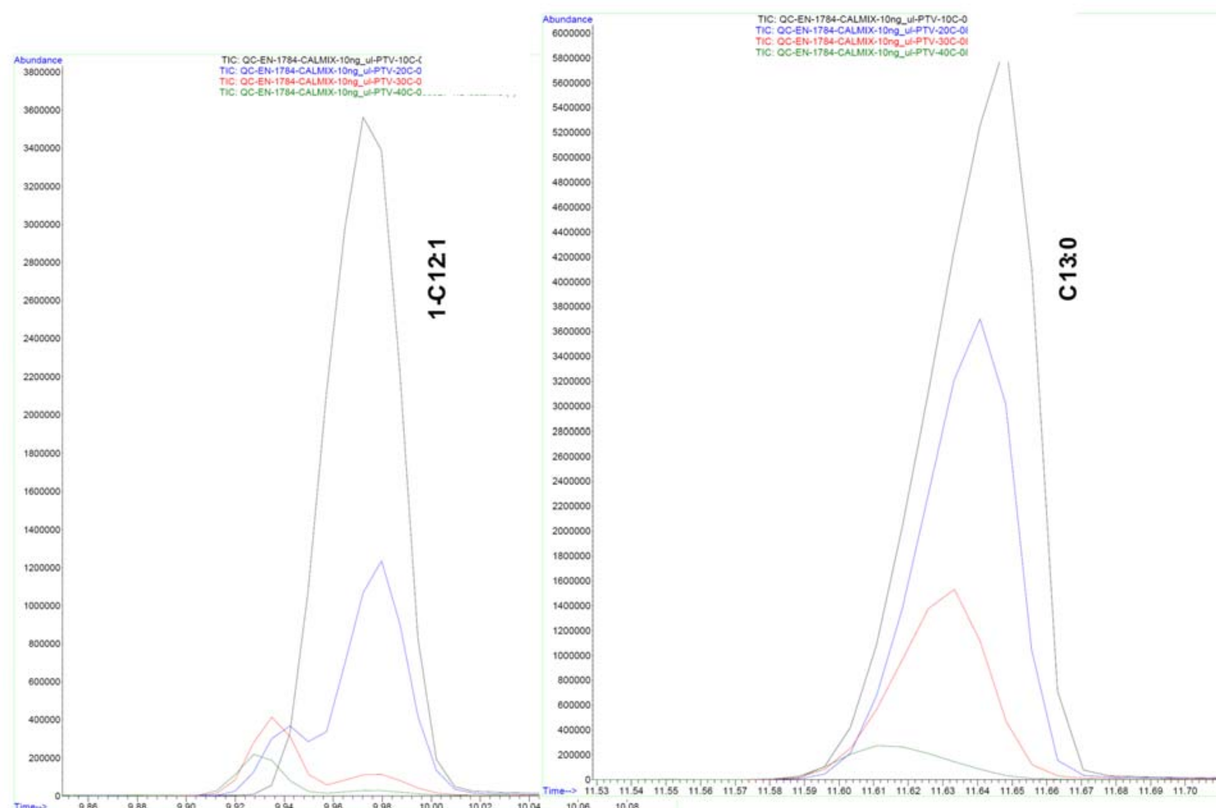

Fig. 1. Solvent vent temperature optimization at 10°C.

Table 1. Fatty acids composition of pure lipids extracted from various types of meat: chicken (literature data [3] and experimental), salmon, crab and shrimp

Fatty acid	Chicken (literature)	Chicken (MIDI)	Salmon (MIDI)	Crab (MIDI)	Shrimp (MIDI)
14:0 (myristic acid)		1	5		2
16:0 (palmitic acid)	21	20	15	7	30
16:1 w7c/16:1 w6c		5	5		7
17:0					4
18:0 (stearic acid)	6	5	3	3	13
18:1 w7c		4	8	1	6
18:1 w9c (oleic acid)	32	32	46	21	17
18:2 w6,9c/18:0 ante (linoleic acid)	25 (18:2 w6,9c)	31	12	66	4
20:4 w6,9,12,15c					6

column separation (HP5-ms column, constant flow at 1 ml/min, carrier gas helium) in a temperature gradient of 15 K/min from 55°C to 300°C [4]. MSD parameters were: transfer line hold constant at 280°C, EI source at 230°C and quadrupole at 180°C, acquisition mode single ion monitoring (SIM) of m/z 98 (dwell 50 ms) and m/z 112 (dwell 50 ms) [4].

Increased sensitivity of 2-ACB chromatographic method consisted in the same optimization of injection parameters as for the HC increased sensitivity chromatographic method for 5 μl (2-ACB extract with internal standard 0.025–2 ng/μl in n-hexane). Capillary column, column flow, temperature program and MSD parameters were the same as for the classic method for comparison purposes.

The ESR spectra of irradiated bones were recorded on an X-band Magnettech MiniScope 200 spectrometer working with a standard rectangular cavity (TE102 mode).

Results and discussion

The MIS RTSBA6 fatty acids profiling method efficiency was checked on chicken fat comparing experimental with the literature data [3].

As it can be seen in Table 1, we have found a very good correlation between the experimental and the literature data (± 1%), the only notable exception being for co-eluted FAME but which can be overcome by GC-MS analysis of the same extract for structural confirmation using AMDIS and NIST'05 library (Fig. 2).

Use of MIS RTSBA6 retention index naming table correlated with the MS spectra of deconvoluted peaks can be a powerful tool for fatty acids identification and composition determination in various pure lipids or even mixtures of different plant and animal origin lipids. Once the fatty acid composition is known, prediction and detection of HC and 2-ACB can be performed [3, 4].

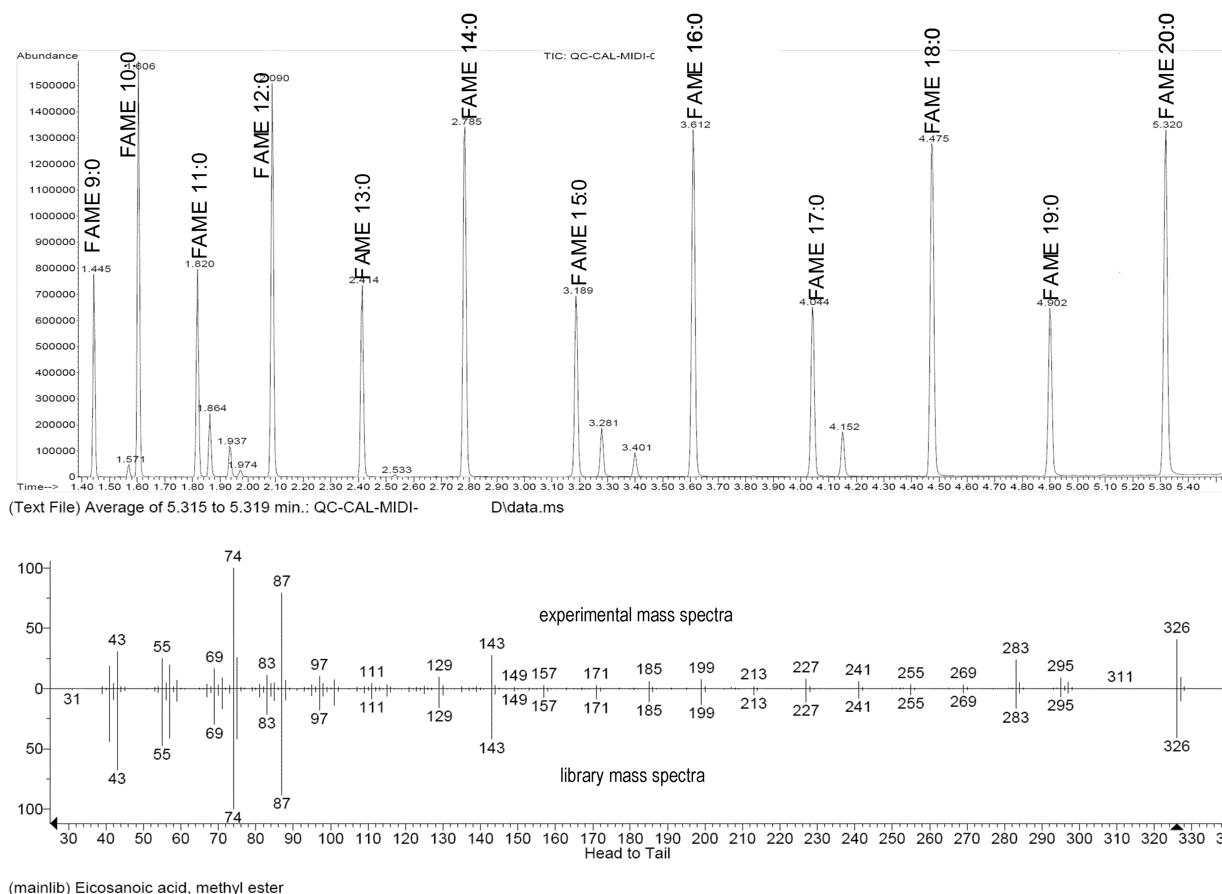

Fig. 2. Example of GC-MS analysis of MIS FAME calibration mixture and MS structural confirmation of FAME C20:0 using NIST 2005 library.

Table 2. Example of main HC and 2-ACB prediction for chicken fat

Fatty acid	%	C*n*–1:0	C*n*–2:1	2-ACB
16:0 (palmitic acid)	20	C15:0	1-C14:1	(2-DCB)
18:0 (stearic acid)	5	C17:0	1-C16:1	(2-TCB)
18:1 w9c (oleic acid)	32	8-C17:1	1,7-C16:2	(2-TDCB)
18:2 w6,9c/18:0 ante (linoleic acid)	25	6,9-C17:2	1,7,10-C16:3	2-tetradaca-5’,8’-dienylcyclobutanone

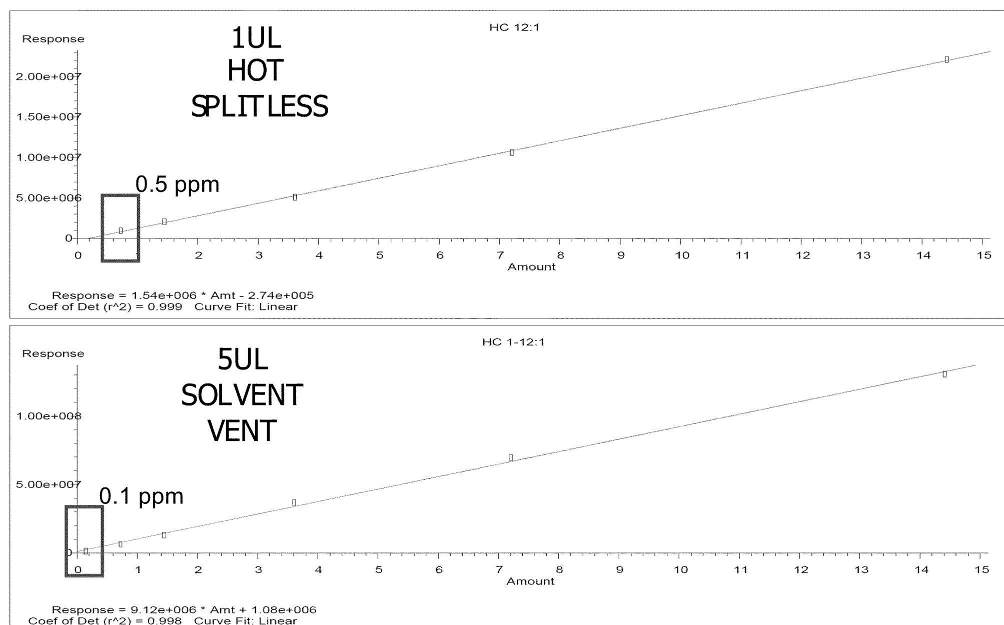

Fig. 3. Calibration curve for 1-dodecene, peak area (TIC – scan from 50–300 amu) vs. target compound concentration, comparison between the standard and modified chromatographic method.

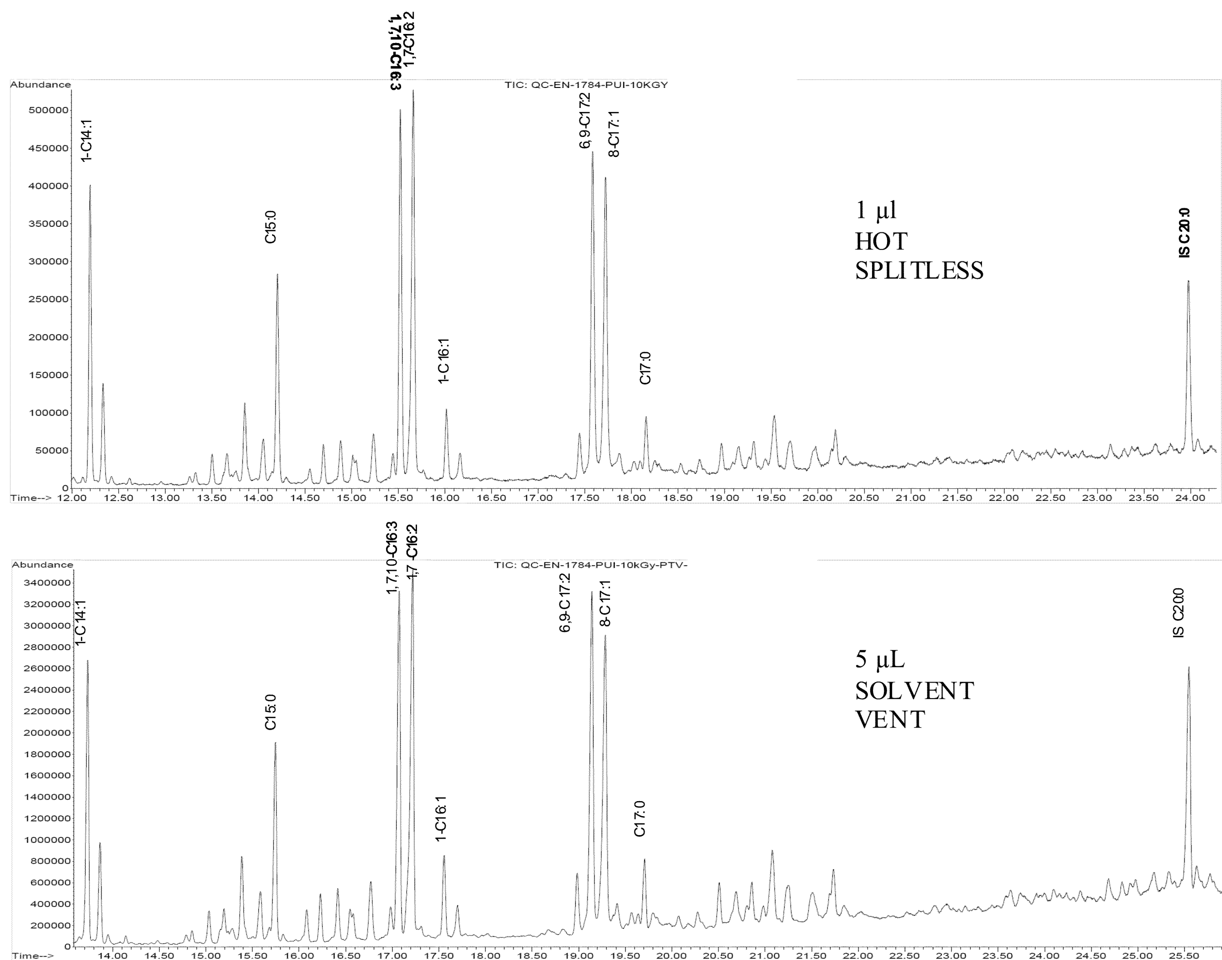

Fig. 4. HC extract from chicken fat irradiated at 10 kGy, standard vs. modified method.

An example of HC and 2-ACB prediction is given in Table 2.

The modified chromatographic method for hydrocarbon detection enabled 10 times smaller concentrations to be detected from 1 to 0.1 ppm as it can be seen in Fig. 3.

HC chromatographic methods were tested for several reference standards for GC including 1-dodecene (1-C12:1), tridecane (C13:0), 1-tetradecene (1-C14:1), pentadecane (C15:0), 1,7-hexadecadiene (1,7-C16:2), 1-hexadecene (1-16:1), hexadecane (C16:0), heptadecane (C17:0), 1-octadecena (1-C18:1), octadecane

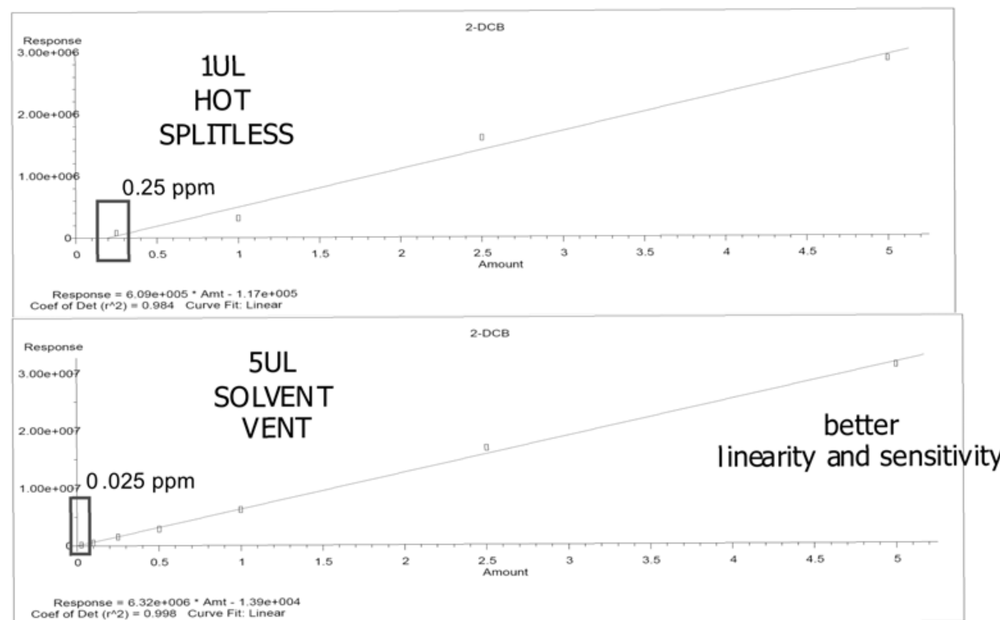

Fig. 5. Calibration curve for 2-dodecylcyclobutanone, peak area (TIC formed by EIC of m/z 98 and m/z 112) vs. target compound concentration, comparison between the standard and modified chromatographic method.

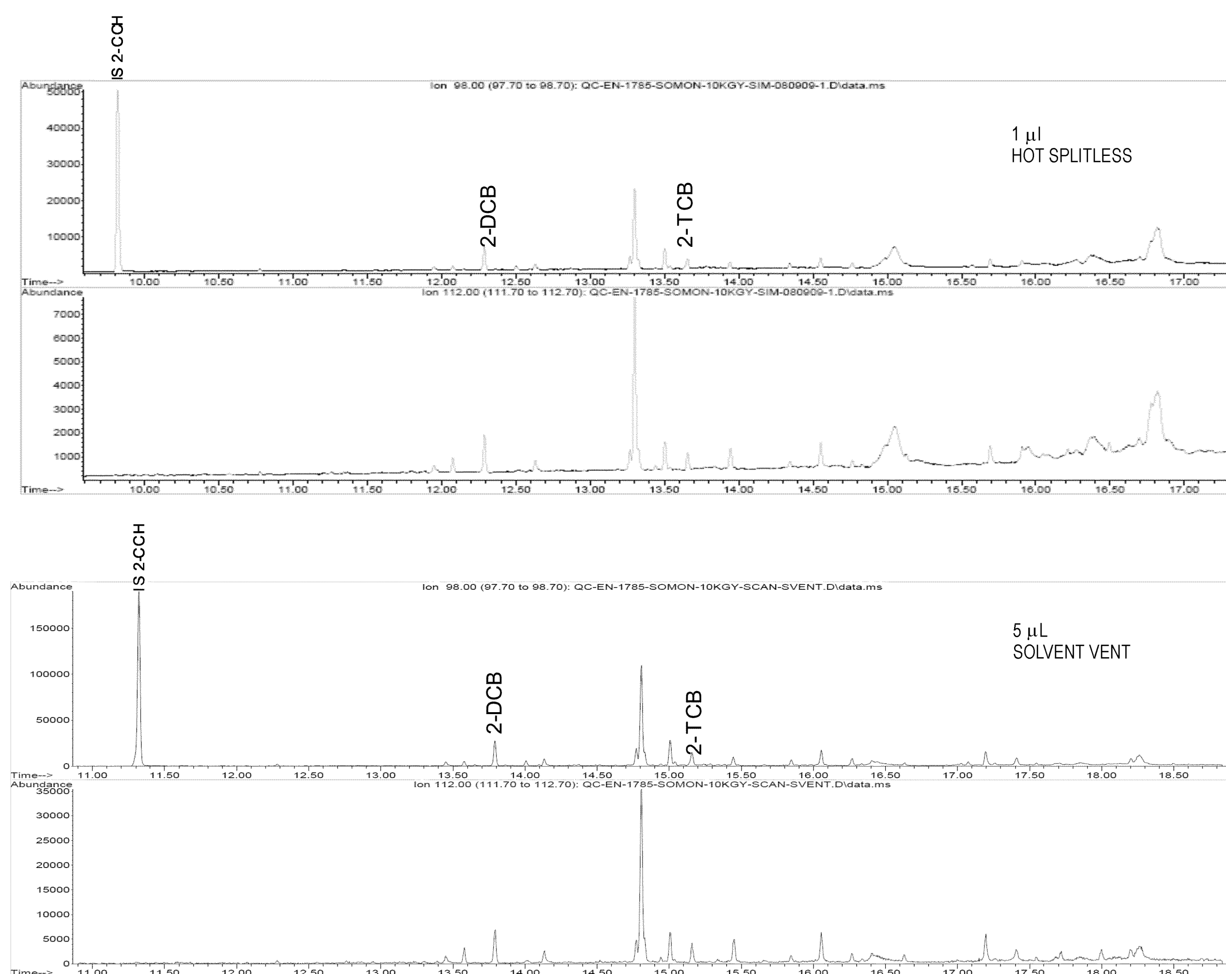

Fig. 6. 2-ACB extract from salmon fat irradiated at 10 kGy, standard vs. modified method.

(C18:0) and eicosane (C20:0) as internal standard.

The sensitivity improvement of at least 7 times in the peak height can be observed in Fig. 4 on the same HC extract obtained for chicken fat irradiated at 10 kGy.

In a similar manner the modified chromatographic method for 2-ACB enables 10 times smaller concentrations to be detected from 0.25 to 0.025 ppm as it can be seen in Fig. 5.

2-ACB standard and optimized methods were tested only for 2-dodecylcyclobutanone (2-DCB) since it was the only available reference 2-ACB in our Laboratory and since the presence of even a single 2-ACB, if detected above 3 times signal-to-noise ratio, is sufficient for declaring the tested sample as irradiated [4]. 2-Cyclohexylcyclohexanone (2-CCH) was used as internal standard.

Similarly to HC, the sensitivity improvement of at least 4 times in peak height can be observed in Fig. 6 on the same 2-ACB extract obtained for salmon fat irradiated at 10 kGy.

Standard extraction procedures involve time and high volumes of solvent, for instance, Soxhlet extraction uses 150 ml/sample for a 6 h extraction [3, 4].

Straight forward extraction techniques like ultrasound fat extraction allow smaller extraction time, simple glassware, with the same or smaller volume of high purity solvents. The direct solvent extraction method [8] enables 3 times smaller solvent volumes, total time reduction of sample preparation to 90 min, and the same results for 2-ACB. Use of last two techniques could lead to comparable results in a more reasonable time per sample and smaller costs.

All ESR spectra of unirradiated samples (chicken, salmon, shrimp and crab) have shown the low intesity symmetrical signal typical of unirradiated samples (Fig. 7).

The typical asymmetric signal attributed to trapped radicals in hydroxyapatite, produced by the action of ionizing radiation on the bone, has appeared in all the ESR spectra of irradiated samples. The amplification used in measurements was the same for all treatment doses of samples.

The shrimp shell was poorly mineralized and non radiation-induced signals have interfered with the radiation specific signal. In the case of crab samples the radiation specific signal has interfered with the signal of Mn^{2+}.

The salmon samples were very fatty and the bones had a poor mineralization, therefore it was difficult to dry and measure them properly.

Conclusions

Fatty acid profiling using Sherlock MIS method [6, 7] is an efficient way of determination fatty acid composition of various fats before HC and 2-ACB detection

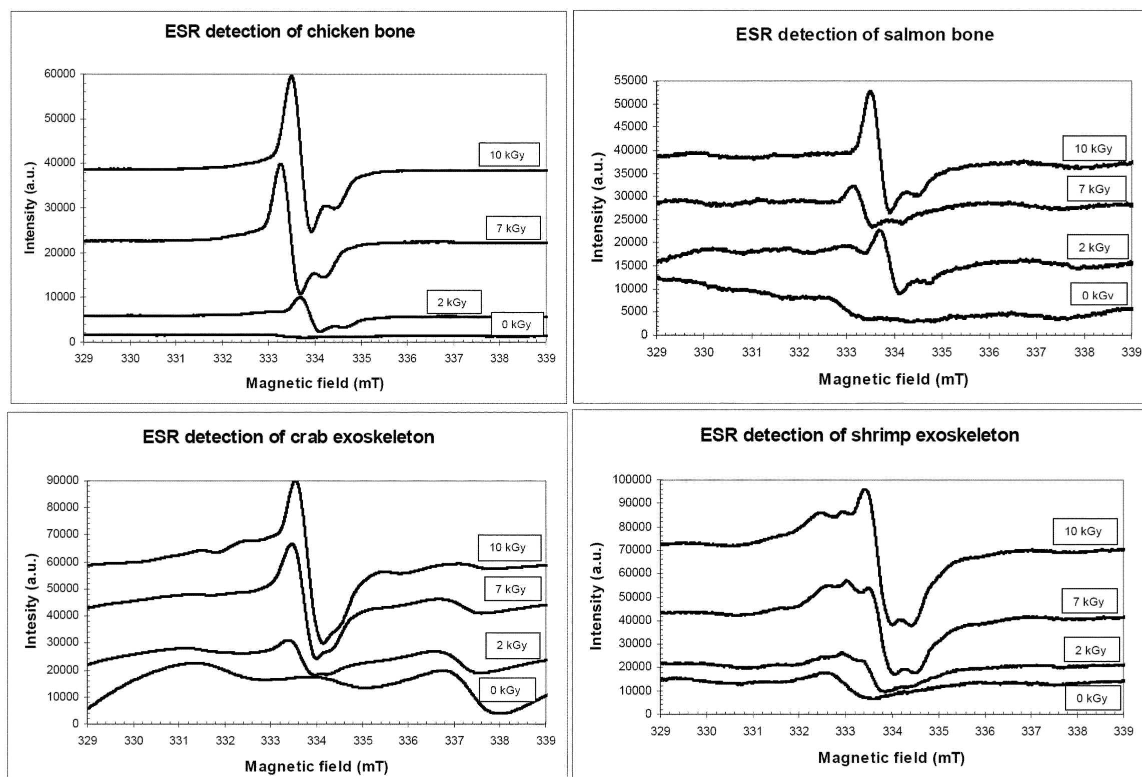

Fig. 7. ESR spectra of chicken and salmon bones and crab and shrimp exoskeleton non-irradiated vs. irradiated at 2, 7, 10 kGy.

enabling use of standardized methods [3, 4] outside of the available validation data.

The ESR method for detection of irradiated foodstuffs containing bone [1] can be used for intercomparison purposes with chemical methods for detection of meat samples. Where bone samples are available ESR is clearly the method of choice for detection of irradiated meat because it is fast and cost effective. However in the absence of bones [1] or mineral residues [2], where fat can be extracted GC-MS methods are the only one able to detect radiation treatment. Use of PTV Cooled Injection Systems can enable detection of smaller quantities of HC and 2-ACB comparing with classical methods. The sensitivity improvement of HC and especially 2-ACB detection in conjunction with faster extraction methods [8] will enable a larger number of samples for routine testing, and maybe smaller analysis costs.

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