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Determination of *trans* fatty acids in foodstuffs by gas chromatography–mass spectrometry after simultaneous microwave-assisted extraction–esterification

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Abstract: A sample preparation method based on the simultaneous microwave-assisted extraction–esterification (SMAEE) was developed for the determination of the fatty acid composition of foodstuffs by gas chromatography–mass spectrometry. The proposed sample preparation method was validated by comparison with the reference Soxhlet extraction method followed by derivatisation by ester formation and the same determination step. The fatty acid compositions and the extraction efficiencies obtained using the proposed SMAEE method and the reference method were statistically similar. The results showed that compared to the conventional method, the SMAEE method offered the advantages of short sample preparation time, low consumption of expensive organic solvents and lower energy consumption. This good agreement between results provided by both the SMAEE and the reference method demonstrates the usefulness of the former as a routine method for the treatment of food samples prior to *trans* fatty analysis.

Keywords: *trans* fatty acids; microwave-assisted extraction–esterification; gas chromatography; mass spectrometry; foodstuffs.

INTRODUCTION

The determination of fatty acid profiles is a basic requirement in the testing of food material as a response to the demand of consumers for improved fat quality in food.[1] Moreover, interest in dietary fat has grown in the last years due to *trans* fatty acids (TFA), which are produced in the hydrogenation process that solidifies liquid oils.[2] Their consumption has been associated with an increase in serum cholesterol levels and the risk of cardiovascular heart diseases. Parallel to

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this, other researchers have reported that *trans* fatty acids decrease the serum levels of the high-density lipoprotein cholesterol.[3–5]

These concerns moved Food and Agriculture, and the World Health Organization to recommend that fats for human consumption should contain less than 4 % of the total fats as *trans* and urged the food industry to reduce the presence of *trans* fats in their products to these levels. The Food and Drug Administration also decreed that by 1 January, 2006, manufacturers must itemize the *trans* fats category separately from the total fat listing. For this purpose, the FDA and Health Canada proposed food-labelling rules that require the amount of *trans* fat per serving to be given. Specifically, products that contain >0.5 g per serving would have the asterisked footnote, "*Includes – g *trans* fat".[6] This is also a concern in Europe, as demonstrated by the Danish legislation that established a lower content of these lipids, < 2 % (w/w), and the general trend in the EU to include the content of *trans* fatty acids on the label as a quality index.[7]

Methods employed to analyse the TFA in foods of natural origin or formed during the processing of fats and oils are of two types. Methods based on infrared spectroscopy only measure the total amount of TFA in a sample, while separation of the different isomers containing one or more double bonds may be achieved only using methods based on gas liquid chromatography (GLC) or high performance liquid chromatography (HPLC). Currently, capillary gas chromatography, with flame ionization (FID) or mass spectroscopy (MS) detectors, is the most appropriate technique for quantifying the composition of fatty acids, including *trans* fatty acids.[8] This separation technique requires the analyte to be volatile, so transesterification to fatty acid methyl esters (FAMEs) is usually performed.

In general, the analytical procedure for the determination of oils or fats from food products comprises three steps: extraction of the fat, esterification and GC analysis. Whereas the last step is completed in 30 to 60 min, extraction takes at least several hours. It is frequently realized by the Soxhlet extraction method, based on iterative percolation of fresh solvent, generally *n*-hexane.[9] After the extraction, most of the solvent is removed in a vacuum rotary evaporator. Much work has been performed to improve the Soxhlet extraction to decrease the operation time and solvent use, and to eliminate the need for evaporation and concentration at the end of the extraction.[10]

Microwave-assisted extraction (MAE) is a relatively novel method of extracting soluble products into a fluid from a wide range of materials using microwave energy. MAE provides a technique whereby compounds can be extracted selectively and in a shorter time compared to those required for conventional extraction methods.[11] MAE supports sustainable development as it consumes less energy than convectional extraction processes while providing in many instance for a reduction in wastes.[12] Liquid-phase MAE is based on the ability of a matrix to absorb microwave energy. This varies with the chemical nature of the species

being exposed to the microwave irradiation. Under MAE conditions, solvents are chosen for their ability to dissolve the target compound and their relative transparency to microwaves. Chemical substances absorb microwave energy at different levels. The parameter generally used as a measure of this physical property is the dielectric constant. Liquid-phase extraction using microwave energy is based on the fact that is possible to immerse the matrix to be extracted in a solvent that is characterized by a small dielectric constant and that is relatively transparent to microwaves.[13] The application of microwave energy as a heat source causes selective heating of the matrix over the extractant. The high, localized temperature and increase in pressure cause a selective migration of target compounds from the material to the solvent at a faster rate and with a similar or better recovery compared with conventional extraction methods.[12]

Recently, much attention has been given to the application of microwave dielectric heating in analytical chemistry because of the reduced analysis time, simplified manipulation and higher purity of the final product. Several classes of compounds, such as fats and oils, essential oils, aromas, pesticides, phenols, dioxins and other organic compounds, have been efficiently extracted from a variety of matrices (mainly soils, sediments, animal tissues, food or plant materials).[14]

The propose of this research was the development of an analytical method for the fast extraction of fat from food with simultaneous derivatisation to FAMEs, based on the use of auxiliary energies, without extract alterations and independent identification–quantification of fatty acids using GC–MS, including the *trans* compounds. Simultaneous microwave-assisted extraction–esterification was used and the obtained results were compared with those of the Soxhlet reference method in order to demonstrate the advantages of the proposed analysis for *trans* fatty acids. On the other hand, GC–MS allows more accurate peak identification to be achieved, fulfils present and future necessities and provides information in relation to the quality of fat used for food elaboration.

EXPERIMENTAL

Instruments

Microwave-assisted extraction was performed in a modified LG 800 W household microwave oven (China) equipped with solvent-extraction equipment. The household microwave oven had to be modified in order to enable connection of an upright reflux condenser located outside the oven with the extraction flask located inside the oven. A hole of 30 mm diameter was drilled at the oven top and 80 mm of the bottom end of an Allihn condenser was passed through the hole and connected to the extraction vessel located in the microwave irradiation zone. Tap water was used as the cooling fluid. A Petri dish (external dimensions: 190 mm diameter, 45 mm high) was placed upside down instead of a rotation plate in order to eliminate rotation of the extraction vessel coupled with condenser during the microwave-assisted extraction process. Polystyrene positioned on the Petri dish was used as a holder for the extraction flask.

A rotary-evaporator (Rotavapor-R, Büchi) was used to evaporate the solvent after Soxhlet extraction.

A Hewlett-Packard HP 5890 gas chromatograph coupled to a HP 5971A quadrupole mass spectrometer equipped with an SP-2560 fused silica capillary column (100 m×0.25 mm, 0.20 μm) coated with highly polar biscyanopropyl polysiloxane liquid phase, provided by Supelco (Bellefonte, PA, USA), was used for the specific analysis of the *trans* fatty acids in the extracts.

Reagents and samples

A multi-standard from Supelco (Cat. No. 47885-U, Bellefonte, PA, USA) containing the methyl esters of 37 fatty acids was used to confirm the retention times and mass spectra for peak identification, as well as to confirm that the peak areas reflected the actual composition of these mixtures. The reagents used were methanol, potassium hydroxide, hydrochloric acid and *n*-hexane. All reagents were of analytical grade purity.

Eleven food items, all commercial, were used in this study. The samples included different bakery and confectionary products, such as caramel, crackers, chocolates, croissant with filling, cookies and biscuits, as these products mainly contain *trans* fats.

Procedures

All the steps involved in the overall analysis, namely, extraction, derivatisation and separation/determination, are described in this section. In all instances, three replicates were made for each sample.

Soxhlet extraction reference method.[15] A homogenized sample (5 g) was weighed into a cellulose extraction cartridge and the Soxhlet apparatus containing the cartridge was fitted to a distillation flask containing 150 ml of *n*-hexane and a few anti-bumping granules. The samples were extracted for 220 min (30–40 cycles/h). After extraction, the solvent was removed using a vacuum rotary evaporator. The total time required was approximately 4 h.

Microwave-assisted extraction. A sample (1.5 g) and 5 ml of *n*-hexane were placed into the extraction flask placed in the microwave oven equipped with an Allihn condenser and irradiated for 5 min at a fixed power of 800 W. After phase separation, a 2.4 cm^3 aliquot of the extract was taken for further preparation of the fatty acid methyl esters.

Simultaneous microwave-assisted extraction–esterification. A sample (1.5 g), 5 ml of *n*-hexane and 1.2 ml of 2.0 mol/dm^3 methanolic potassium hydroxide solution were placed into the extraction flask placed in the microwave oven furnished with an Allihn condenser. After leaching (5 min of microwave irradiation at 800 W), 2.4 ml of 1.0 mol/dm^3 HCl was added and gently stirred. After phase separation, the upper phase containing the fatty acid methyl esters was decanted off and finally a 1.0 μl aliquot was used for GC–MS analysis. The total time required was approximately 15 min.

Preparation of fatty acid methyl esters. After Soxhlet extraction, approximately 150 mg of fat extract was put into a test tube and dissolved in 2.4 cm^3 of *n*-hexane, while after microwave-assisted extraction, an aliquot of 2.4 cm^3 of *n*-hexane phase was taken. Further, the extracts were treated in the same way. An aliquot (0.60 cm^3) of 2.0 mol/dm^3 methanolic KOH solution was added. The tube was capped and vigorously shaken for 20 s and allowed to boil for one minute in water bath at 70 °C. After 20 s of shaking, 1.2 cm^3 of 1.0 mol/dm^3 HCl was added and gently stirred. After phase separation, 3 cm^3 of *n*-hexane was added and the upper phase containing the fatty acid methyl esters was decanted and dissolved in *n*-hexane to 5.0 cm^3. Finally, 1.0 μl of the thus-obtained solution was injected into the GC–MS.

Gas chromatography–mass spectrometry analyses

Helium at a constant flow rate of 0.58 cm^3/min was used as carrier gas for the GC–MS analysis of the FAME extracts. The following temperature program was used: injector temperature 230 °C, initial column temperature 100 °C (held 5 min), temperature ramp 10 °C/min to 240 °C and held at this temperature for 10 min. The total run time was 30 min. The injection was performed manually, volume 1.0 µl, with a split ratio 1:80.

The mass spectrometer was operated in the electron ionization mode with a quadrupole temperature of 180 °C. Data acquisition was realised in the scan mode (range 40–400 m/z). The instrument was tuned daily by operating the software programs (Autotune) using perfluorotributylamine (PFTBA) as the calibration substance. Mass spectrometer parameters were adjusted so that the masses 69, 219, and 502 and their respective isotopes met the target mass – intensity criteria.

The fatty acids were identified by comparing their retention times and mass spectral data to the mass spectral data obtained by analysis of standard fatty acid methyl esters solution under the same conditions. A commercial database of mass spectra "Wiley" was also used.

The response factor, mean of five injection of the standard solution for each fatty acid methyl ester present in the calibration standard solution, was calculated related to palmitic acid according to Eq. (1):

$$R_i = \frac{m_{0,i} A_{16:0}}{m_{16:0} A_{0,i}} \quad (1)$$

where $m_{0,i}$ is the mass % of $FAME_i$ in the calibration standard solution; $A_{16:0}$ the peak area of 16:0 in the calibration standard solution chromatogram; $m_{16:0}$ the mass % of 16:0 in the calibration standard solution; $A_{0,i}$ the peak area of $FAME_i$ in the calibration standard solution.

The content of each fatty acid expressed by mass percentage was calculated according to relation (2):

$$100 \frac{R_i A_i}{\sum R_i A_i} \quad (2)$$

where R_i is the response factor for each fatty acid and A_i the peak area of the fatty acid methyl ester in the sample solution.

RESULTS AND DISCUSSION

Different microwave-assisted extraction methods have already been proposed for the extraction of the total fat content from a variety matrices, such as food,[9,14] cocoa powder and cocoa nibs,[11] seeds[16] and poultry feeds.[17] MAE has also been suggested for the determination of the fatty acids profile.[7,8,18,19] However, the applicability of the simultaneous microwave-assisted extraction–esterification method for the determination of the fatty acids profile with emphasis on *trans* fatty acids has not been demonstrated.

Optimization of simultaneous microwave-assisted extraction–esterification

When two-step methods are studied, it is difficult to separate the extraction and esterification effects. For this reason, microwave-assisted extraction followed by esterification was compared with classical Soxhlet extraction followed by es-

terification in terms of extraction efficiency. The main factors affecting microwave-assisted extraction in open vessel systems are: solvent nature, extraction time and microwave power. The choice of solvent for MAE is dictated by the solubility of the target analyte, by the interaction between the solvent and matrix, and finally by the microwave absorbing properties of the solvent.[20] Another important aspect is compatibility of the extracting solvent with further chromatographic analytical steps.[21]

The effect of microwave energy is strongly dependent on the nature of both the solvent and the solid matrix. Solvents generally used cover a wide range of polarities, from heptane to water. The microwave-assisted extraction process may occur by a number of mechanisms: the sample could be immersed in a single solvent or mixture of solvents that strongly absorb the microwave energy (mechanism I); the sample could be extracted in a combined solvent containing solvents with both high and low dielectric losses mixed in various proportions (mechanism II); samples that have a high dielectric loss (*e.g.*, with a high water content) can be extracted with a microwave transparent solvent (mechanism III).[22]

It was demonstrated that lipid extraction is highly dependent on the solvent used and, consequently, the choice of solvent is one of the most critical decision in the determination of fat.[16] Thus, the solvent used in all the extraction methods was that proposed in the ISO method, *i.e.*, *n*-hexane.[23] Considering the fact that the main goal of this study was the development of a fast sample preparation method for the determination of the fatty acid composition of foodstuffs by GC–MS, the use of more polar solvents in the microwave-assisted extraction was rejected to avoid differences between the compositions of the extracts and solvent exchange before derivatisation or the chromatographic step.

Most times, the chosen solvent for microwave-assisted extraction possesses a high dielectric constant and strongly absorbs microwave energy, however, the extracting selectivity and the ability of the medium to interact with microwaves can be modulated by the use of a mixture of solvents. In some cases, as in this study, the matrix itself interacts with the microwaves while the surrounding solvent possesses a low dielectric constant and thus remains cold. This is possible since the microwaves interact selectively with the polar water molecules naturally present in foodstuffs. Localized heating leads to the expansion and rupture of the cells, causing a rapid and selective expulsion of the fat into the relatively cool surrounding solvent that solubilises it rapidly. With samples having a high dielectric loss, efficient extractions can be performed using pure, microwave-transparent solvents (mechanism III).[22]

In order to avoid extract evaporation before the derivatisation step, factors such as the amount of solvent in contact with the sample when the microwave irradiation is applied and the amount of sample were fixed to constant values. The

amounts of sample and solvent were calculated in relation to the fat content; hence, the extract contained approximately 150 mg per 2.4 ml *n*-hexane.

Microwave power and irradiation time are two factors that influence each other greatly. Preliminary experiments showed that efficient extraction could not be achieved if the MAE process was stopped before the commencement of rapid boiling of the extraction mixture. The microwave-assisted extraction process was performed with the irradiation power set at 160 (minimum level), 480, 640 and 800 W (maximum level), whereby rapid boiling of the reaction mixture began at 12, 6, 4 and 1.5 min, respectively. In order to achieve an efficient extraction in the shortest possible time, the maximal microwave power (800 W) was selected as the optimal and used for subsequent experiments. The extraction time was changed from 2 to 12 min, in order to determine the optimal time. The results (Fig. 1) indicate that amount of fat extracted initially increased with increasing extraction time, but that a plateau was reached after 5 min, the four longer extractions gave similar analytical signal.

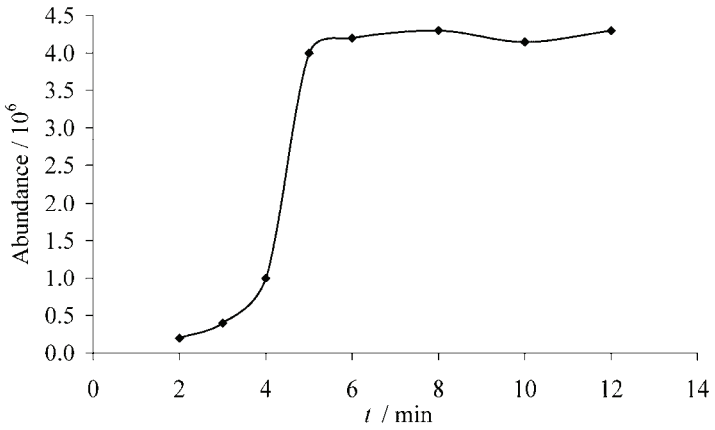

Fig. 1. The dependence of average analytical signal on the extraction time.

In view of these results, 5 min was selected as the optimal extraction time and used for further experiments. The accepted optimal working conditions were those described under “microwave-assisted extraction” and “simultaneous microwave-assisted extraction–esterification”.

Comparison of the composition of the extract obtained by the different method

The optimal working conditions obtained for the proposed method were applied for all samples under study and the results compared with those provided by the reference Soxhlet method in terms of extraction efficiency. The average extraction efficiencies obtained by the three methods provided by each analyte are given in Tables I and II (Supplementary material).

A two-tailed *t*-test was used to compare the means of related (paired) samples in order to evaluate if microwave-assisted extraction followed by esterification and simultaneous microwave-assisted extraction–esterification yielded similar results as Soxhlet method at the 95 % confidence level. The null hypothesis was that both methods yielded the same results or, in other words, that the observed differences between the Soxhlet and the MAE or SMAEE methods were not significant. The calculated *t*-values were compared with the theoretical value at $\alpha = 0.05$ and suitable degrees of freedom. As the calculated values were smaller than the theoretical value, the null hypothesis was accepted. This means that at the chosen significance level, the differences between the values obtained for the different fatty acids were within experimental error. Particularly, the standard deviation for all compounds ranged between 0.00 and 1.80, see Tables I and II. As can be seen, similar extraction efficiencies were provided by both the proposed simultaneous microwave-assisted extraction-esterification method and the Soxhlet reference method. Furthermore, the similar extraction efficiency of *trans* compound indicates that alterations of the double bonds did not occur. The advantages of SMAEE *vs*. Soxhlet extraction (Table III), such as: a drastic reduction of both the procedure time and sample handling, 15 min *vs*. 4 h, and the smaller amount of organic solvent required, make the SMAEE method an alternative with reliable possibilities for replacing the Soxhlet method in routine analysis in view of the imminent policy of mandatory characterisation of the fat content in foodstuffs.

TABLE III. Comparison of the optimal conditions of the simultaneous microwave-assisted extraction–esterification (SMAEE) method with the Soxhlet reference method

Condition	Soxhlet extraction	SMAEE
Solvent volume, ml	150	5
Total time, min	240	15
Special	Extraction 220 min (30–40 cycles/h); solvent evaporation: 10 min; FAMEs preparation: 10 min	–

CONCLUSIONS

Fatty acid analysis, with special emphasis on TFA, was performed on eleven food samples using a very fast and effective sample preparation method – simultaneous microwave-assisted extraction–esterification. The proposed method was compared with the reference method – Soxhlet extraction followed by esterification. Gas chromatography–mass spectrometry was used for individual separation and detection. Similar extraction efficiencies were provided by both the proposed SMAEE method and the Soxhlet reference method. The fatty acid composition obtained by the use of the simultaneous microwave-assisted extraction–esterification method and the Soxhlet reference method can be regarded as statistically equivalent. The SMAEE method provides a substantial reduction in the

sample preparation time (from approximately 15 min to 4 h) with a minimal solvent requirement relative to Soxhlet extraction followed by derivatisation.

The results of this study suggest that the presented SMAEE method could be appropriate for routine quality control analysis of fats in food products.

SUPPLEMENTARY MATERIAL

The average extraction efficiencies obtained by the three methods (Tables I and II) are available electronically from http://www.shd.org.rs/JSCS/ or from the corresponding author on request.

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ИЗВОД

ОДРЕЂИВАЊЕ *trans* МАСНИХ КИСЕЛИНА У ПРЕХРАМБЕНИМ ПРОИЗВОДИМА ГАСНОМ ХРОМАТОГРАФИЈОМ–МАСЕНОМ СПЕКТРОМЕТРИЈОМ НАКОН ИСТОВРЕМЕНЕ МИКРОТАЛАСНЕ ЕКСТРАКЦИЈЕ И ЕСТЕРИФИКАЦИЈЕ

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У оквиру овог рада развијена је метода за припрему узорака у циљу одређивања састава масних киселина у прехрамбеним производима гасном хроматографијом–масеном спектрометријом заснована на истовременој микроталасној екстракцији и естерификацији (SMAEE). Валидација методе је изведена поређењем са резултатима добијеним гасном хроматографијом–масеном спектрометријом након екстрације по Soxhlet-у и дериватизације масних киселина у метилестре масних киселина. Резултати добијени применом предложене и референтне методе били су статистички исти, како у погледу састава масних киселина, тако и ефикасности екстракције. Резултати су показали да су предности SMAEE у односу на конвеционалну методу следећи: кратко време припреме узорка, и самим тим мања потрошња енергије, као и употреба малих количина скупих органских растварача. Добро слагање резултата добијених применом референтне и методе засноване на истовременој микроталасној екстракцији и естерификацији показује да би се SMAEE могла применити као рутинска метода за припрему узорака прехрамбених производа у циљу одређивања *trans* масних киселина.

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