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Automatic microgravimetric determination of fats in milk products by use of supercritical fluid extraction with on-line piezoelectric detection

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Abstract

An on-line supercritical fluid extraction-piezoelectric detection system was developed and applied to the quantitative gravimetric determination of total fat in food samples (skimmed milk and cocoa). The proposed assembly provides all the advantages of an on-line system as regards automation, in addition to acceptable sensitivity and precision. Its strength lies in the design of the interface between the supercritical fluid extractor and the piezoelectric detector. Samples of skimmed milk and cocoa are weighed in the extraction thimble, previously loaded with 1 g of diatomaceous earth. A temperature of 100°C and a CO_2 fluid density of 0.60 mg/ml are used for extraction. The linear calibration range thus achieved is 0.005–0.07% w/w total fat, and the relative standard deviation is ±2.3% ($n=11$; $P=0.05$). The throughput is six samples h^{-1} (for the overall process). The proposed method was used to determine the total fat in food samples (milk, cocoa), the results being competitive with those of the Soxhlet methods for the same purpose.

Keywords: Piezoelectric detection; Microgravimetry; Food samples; Fats

1. Introduction

The determination of fat content is of special interest to industrial food processors. This control is indispensable in order to meet, the increasing consumer demands for products containing less fat (i.e., ''fat free'' or ''low-fat'' foods), as well as to maintain costs, comply with labeling regulations and with the resulting increasingly stricter analytical requirements. Methods for fat determination must assure accurate labeling of food products. Although the determination of fat content is one of the more common analyses, traditional methods for extracting fats involve long extraction times and high consumption of solvents. In addition, the solvents are potentially hazardous to the environment and expensive [1,2]. Alternative methods using organic solvents more sparingly are therefore required. King and Eller [3] investigated supercritical fluid extraction (SFE) as an alternative to solvent-based extraction methods. Subsequently, Snyder et al. [4] developed a method for the rapid determination of nutritional fat content in meat samples. Additional research about supercritical fluid extraction for the determination of total

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fat and lipid classes in meats was conducted by Berg et al. [5], who compared their results with those of conventional methods. The previous methods provide better extraction efficiency than other, conventional techniques. Additional advantages of SFE include shorter extraction times and the ability to implement SFE in an on-line configuration. Although SFE has a high potential for off-line sample preparation, it requires the active involvement of the operator, decreases the precision of the results and is subject to analyte losses in trace analysis [6]. Various designs have been developed to meet the determination needs for specific analytes. Thus, Dressman and Michael [7] used on-line electrochemical detection with supercritical fluid chromatography to determine hydroquinone compounds. Fuoco et al. [8] determined organic pollutants in enviromental samples by using a SFE system coupled on-line with gas chromatography–mass spectrometry (GC–MS). A similar SFE-GC–MS design was used by Ramsey et al. [9] to determine phenols in water. Taylor and Jordan [10] reviewed the applications of supercritical fluid extraction coupled directly with Fourier transform infrared spectrometry. An fiber-optic interface for on-line supercritical fluid extraction was developed for Heglund et al. [11] for the determination of total petroleum hydrocarbons in soil. PCBs in biological samples were determined with recoveries between 70 and 96% by Johansen et al. [12]. Using determinations an on-line SFE-GC system of fat content in foods poses a challenge to the design of methods that provide a rapid accurate response. Berg et al. [13] studied the separation and quantitation of edible components fat by using SFE coupled on-line with SFC. Lieschesk [14] coupled detection infrared spectroscopy on-line with SFE to determine the total lipid content in milled rice flours. Most SFE methods for the determination of total fat are gravimetric [15–22].

The piezoelectric quartz crystal (PZ) is very sensitive to mass changes [23–25], so and it can be used as a microbalance in microgravimetric applications [26,27]. A number of analytical applications of the PZ have recently been reported [28–31]. In this work, a readily automated on-line coupled SFE-PZ system for the quantitative determination of total fat was developed.

2. Experimental

2.1. Standards, chemicals and solvents

A stock standard solution containing 1% palmitic acid was prepared by dissolving 1 g of the product (Sigma Ultra) in 100 ml of *n*-hexane (Scharlau, Hight Purity). Standard working solutions were prepared by appropriate dilution of the stock in *n*-hexane. Diatomaceous earth (acid-washed, approximately 95% SiO_2) was purchased from Sigma and used as received. SFC-grade carbon dioxide from Air Liquide (Paris, France) was used as extraction fluid. All solvents were HPLC-grade and previously filtered.

Working standards were obtained by spiking. First, 1 g of diatomaceous earth was weighed in the extraction thimble, and then appropriate volumes of the standard working solutions were added to the diatomaceous earth in the thimble. Finally, the spiked solid was allowed to stand for at least 12 h in order to allow *n*-hexane to evaporate.

2.2. Apparatus

2.2.1. Piezoelectric detector

An At-Cut 10 MHz piezoelectric quartz crystal (14 mm diameter×0.17 mm thick) coated with gold plated electrodes on both sides was used. The quartz crystal was housed in a flow-through PEEK™ cell and clamped between two O-rings recessed into the housing (Fig. 1); one crystal face was exposed to the sample in a cell of 70 μl. The piezoelectric end PEEK™ was supplied by Universal Sensors, Inc. (Metairie, LA, USA). A laboratory-made oscillator circuit was connected to the electrode via platinum foil; the resonant frequency was monitored with a Hewlett-Packard HP-53181A/225 MHz frequency counter that was connected to a PC-pentium® microcomputer via an HP-IB interface (Hewlett-Packard). HP-34812A BenchLink software (HP BenchLink/Meter) was used to acquire and store data. A Gilson Minipuls-3 peristaltic pump and a Rheodyne 5020 injection valve were also used.

2.2.2. Supercritical fluid extraction

All SFE experiments were performed on 7680T

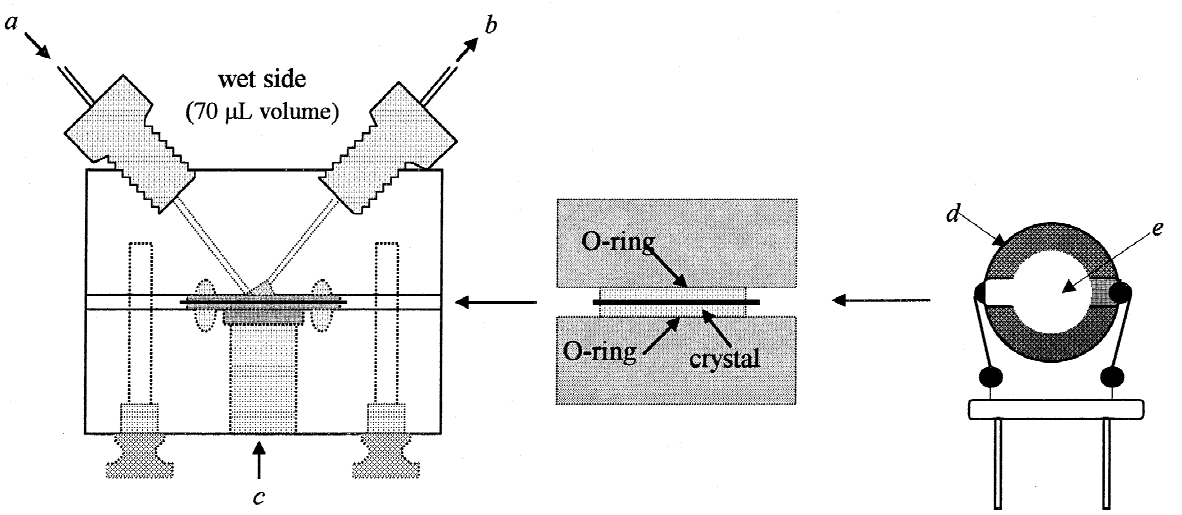


Fig. 1. Detection system. Flow-cell: (a) inlet, (b) outlet, (c) dry side, (d) 10 MHz piezoelectric crystal, including 14 mm diameter×0.17 mm thick quartz disc and (e) 7.8-mm diameter gold electrode.

Hewlett-Packard supercritical fluid extractor equipped with a Hewlett-Packard 1050 isocratic modifier pump, furnished with a 7-ml extraction vessel, an automated variable restrictor, a stainless steel trap and a solid-phase trap packed with Octadecylsilica (ODS). Extractions were conducted in 7-ml thimbles. Each extraction was done in triplicate, so the recoveries given are the averages of three extractions each. Samples were subjected to dynamic extraction for 5 min. A static extraction period of 2 min was used. All extractions were carried out at 256 bar, using CO_2 as the fluid. The extraction temperatures were 100°C. The extracted analyte was collected in a stainless steel trap. Upon completion of each extraction, the analyte was eluted from the trap at 45°C with 1.0 ml of *n*-hexane.

2.2.3. On-line supercritical fluid extraction-piezoelectric detection

The hardware and operating procedures described in the SFE and piezoelectric detection (PZ) sections were used to couple SFE on-line to PZ. A manifold interface was constructed for this purpose. Fig. 2 depicts the system used and Fig. 3 shows the location of the interface, which consisted of a stainless steel junction 2.5 cm long×250 μm I.D. One end of the junction was connected to the trap outlet and the other to the manifold.

Once the extraction was complete and the SFE system was decompressed to atmospheric pressure, the valve IV was switched to its filling position. The SFE rinse pump propelled the eluent through the interface. At an appropriate time (the elution time of the analyte of interest, determined by processing standards), the fraction containing the target analyte was injected by means of injection valve IV. The fraction of interest held in the sample loop (350 μl) was driven by the *n*-hexane carrier to the piezoelectric flow-cell (FC-PZ), where the signal was detected, transmitted to the frequency counter (F) and recorded (PC). Samples and calibration standard solutions were injected into the flow once the base resonant frequency (F_b) levelled off and measurements were found be $\Delta F = F_p - F_b$, where F_p is the maximum frequency in each run.

A displacement flask (DF) was used to avoid passage of the organic solvent through the pump tubes. A step valve (SV) was employed to recover the clear solvent.

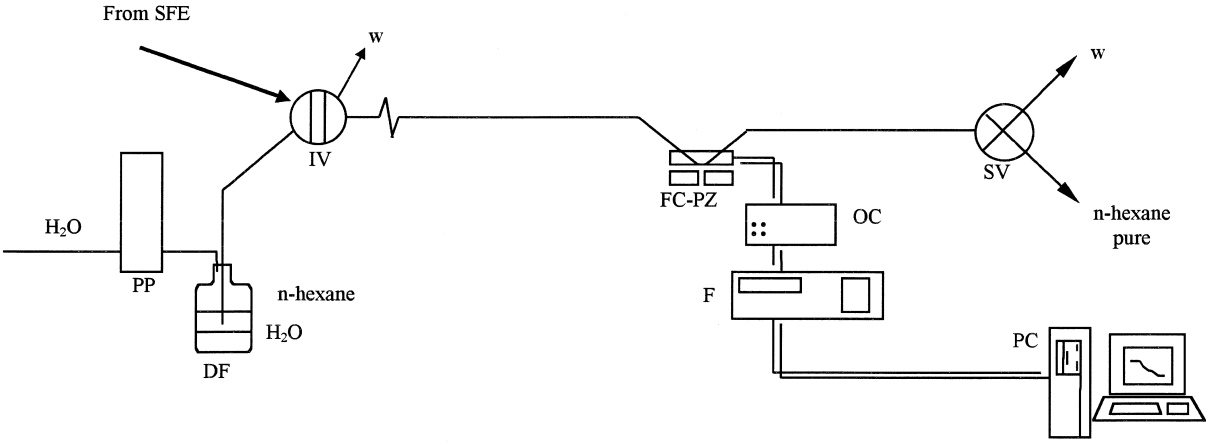


Fig. 2. Manifold (interface) for the determination of total fat content in foods (milk, cocoa). PP = Peristaltic pump, IV = injection valve, DF = displacement flask, w = waste, FC-PZ = flow cell-piezoelectric crystal, SV = step valve; OC = oscillator circuitry, F = frequency counter, PC = interfaced personal computer.

3. Results and discussion

Fig. 4 shows the typical changes in oscillation frequency for an injection of a 0.02% w/w standard of palmitic acid. The frequency decreased at the face of the crystal into contact with the carrier (*n*-hexane) containing the analyte. The fast response of the crystal allows the analyte concentration to be determined within a few seconds. For microgravimetric application, a quantitative relationship between the relative shift in the resonant frequency and the added mass (fat matter extracted) must be established. The Sauerbrey equation [23–25], which is the fundamental relation for the piezoelectric detector, is only of semiquantitative value. It was therefore necessary to use calibration curves for quantitation, and avoid factors such as mechanical clamping and damping in the electrical circuit [25]. Viscosity and density remained unchanged as the temperature was kept constant (22±1°C). This suggest that the frequency change is caused by the small mass change in the quartz electrode surface [26], and hence that it depends on the analyte concentration alone. In fact, the frequency change (detector response) was found to be linearly related to the concentration of the injected palmitic acid standard. The response of the piezoelectric detector was stable over long periods and easy to maintain an unavoidable requirement in routine analyses.

3.1. Optimization of the SFE conditions

Preliminary experiments showed that the extraction yield of fat depended on the extraction, collection and elution conditions. Therefore, the influence of all these experimental conditions was initially considered.

3.1.1. Influence of the CO_2 density and pressure

Fig. 5a shows the effect of altering the density, at a constant temperature (80°C), on the recovery and pressure. Increasing densities up to 0.60 g/ml considerably increased fat extraction (>95%). Such favourable results can be due to solute–solvent interactions in heavy CO_2, increasing the solubility of fats and the rise in pressure increases the density. The solvent power of a supercritical fluid (CO_2) at a given temperature increases with increase in density.

3.1.2. Influence of the extraction temperature and pressure

The effect of the extraction temperature on fat recovery was also studied. The effect of temperature constant CO_2 density (0.60 g/ml) on the recovery

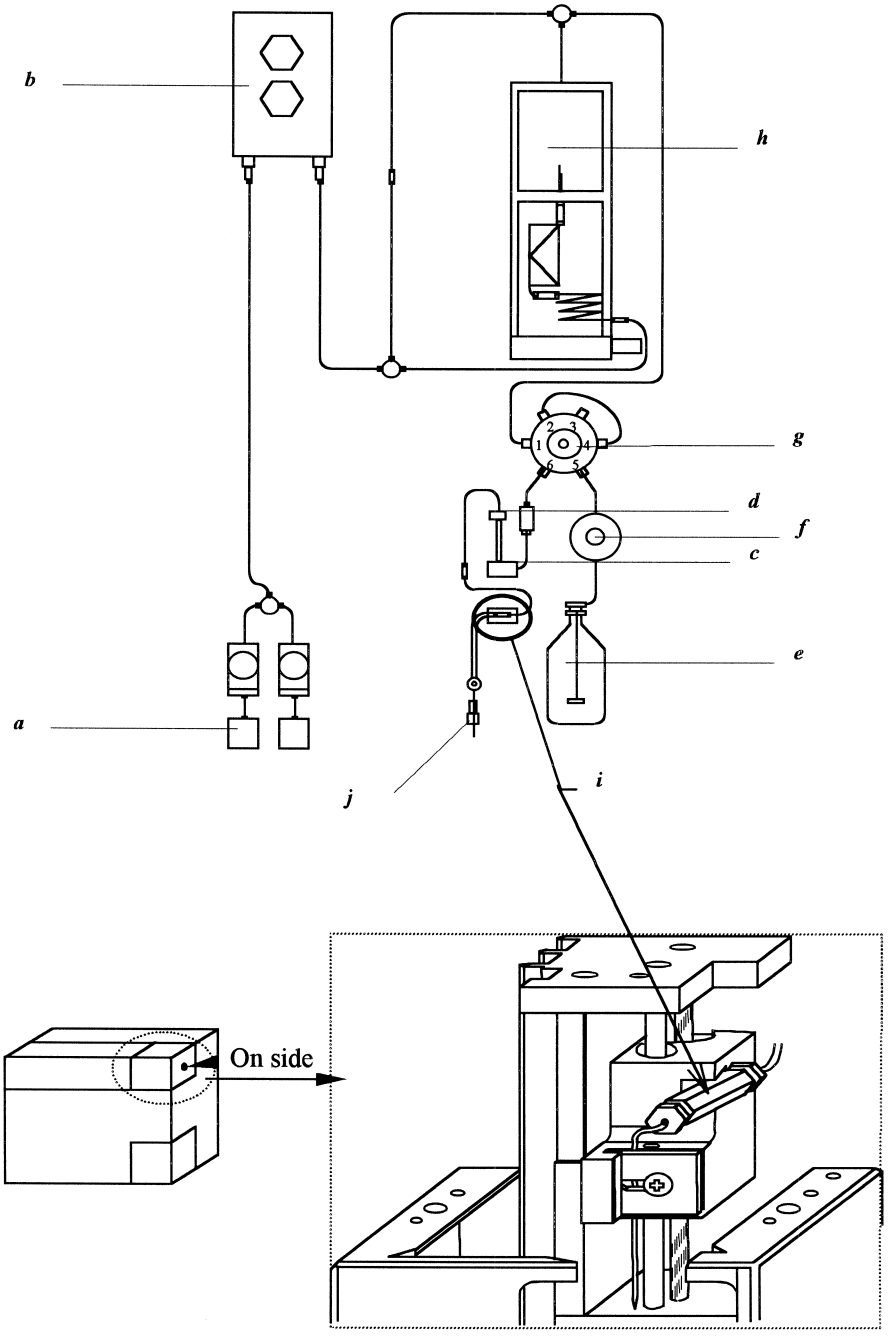


Fig. 3. Location of the interface in the on-line SFE system. (a) Fluid extraction valve, (b) fluid extraction pump, (c) nozzle (restrictor), (d) trap, (e) rinse solvent, (f) rinse pump, (g) six-port valve, (h) extraction chamber, (i) location of the interface, (j) waste and (k) to the PZ detector.

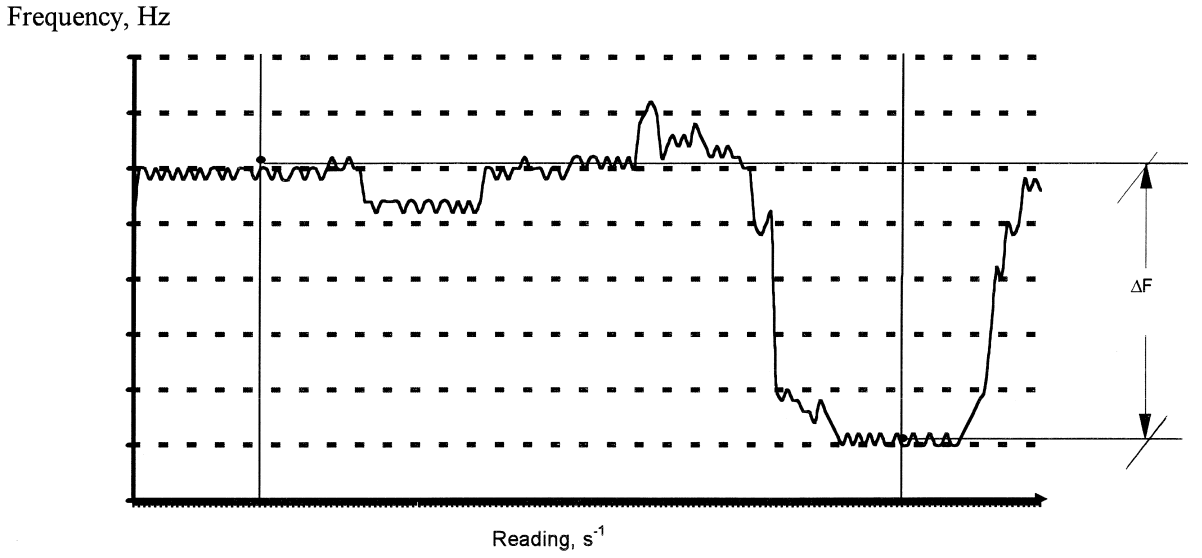
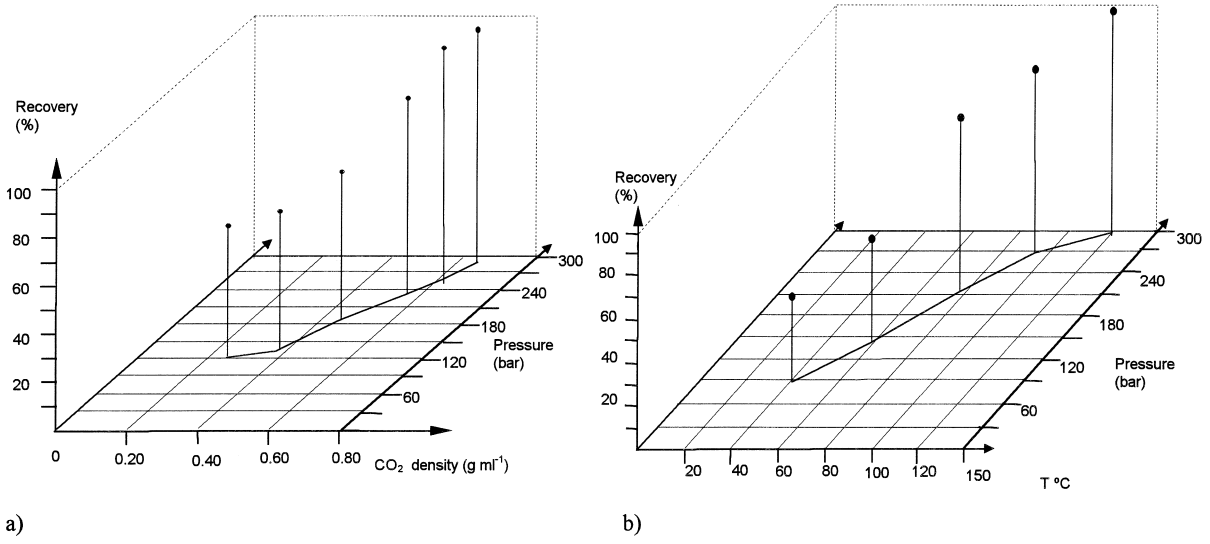


Fig. 4. Frequency vs. reading (time response) plot for a 0.02% w/w palmitic acid standard. Flow-rate = 0.4 ml/min, injected volume = 350 μl.



a) b)

Fig. 5. (a) Influence of the CO_2 extraction pressure on the SFE efficiency for fat in foods (milk, cocoa). Amount of sample = 0.01 g; SFE conditions: CO_2 flow-rate; 1 ml min^{-1}; extraction temperature = 100°C; stainless steel trap; nozzle (restrictor) and trap temperature during collection = 70 and 55°C, respectively; rinse solvent = *n*-hexane; rinse volume = 1 ml; nozzle and trap temperature during rinse = 60 and 45°C, respectively; % recovery of fat matter is expressed as % w/w of palmitic acid. (b) Effect of the extraction temperature on the SFE efficiency for fat from foods (milk,cocoa). Amount of sample = 0.06 g; SFE conditions: CO_2 flow-rate = 1 ml min^{-1}; extraction temperature = 100°C; stainless steel trap; nozzle (restrictor) and trap temperature during collection = 70 and 55°C, respectively; rinse solvent = *n*-hexane; rinse volume = 1 ml; nozzle and trap temperature during rinse = 60°C and 45°C, respectively; % recovery of fat matter is expressed as % w/w palmitic acid.

and pressure is illustrated in Fig. 5b. The optimal temperature was 100°C. The solvent power of a supercritical fluid at a given density increases with increasing temperature, this is reflected in the substantial recovery of fats (>98%). The pressure was dictated by the previous two parameters (the working pressure value was 256 bar), which is low enough to ensure a long lifetime of the SFE equipment.

3.1.3. Extraction time

Extraction tests were conducted to optimize the extraction time. The effect of this variable is shown in Fig. 6. As can be seen, the influence of the extraction time on fat recovery was critical up to 5 min. An extraction time of 7 min slightly increased the percentage recovery; however, a short time was suitable for implementing screening methods. The combination of an initial static period with a subsequent dynamic one was used to improve this extraction approach. During the static period, the chemical bond between the analyte and the sample matrix was broken and a single dynamic extraction provide a recovery of about 90%; the static–dynamic protocol gave an even higher recovery (>98%).

3.1.4. Influence of the modifier and void volume

The influence of ethanol as the CO_2 modifier was examined. The alcohol was added to the preweighed sample in amounts of 10–30%. The results were essentially similar to those obtained in the absence of modifier. This can be ascribed to the extremely high solubility of fats and oil in supercritical fluids [32].

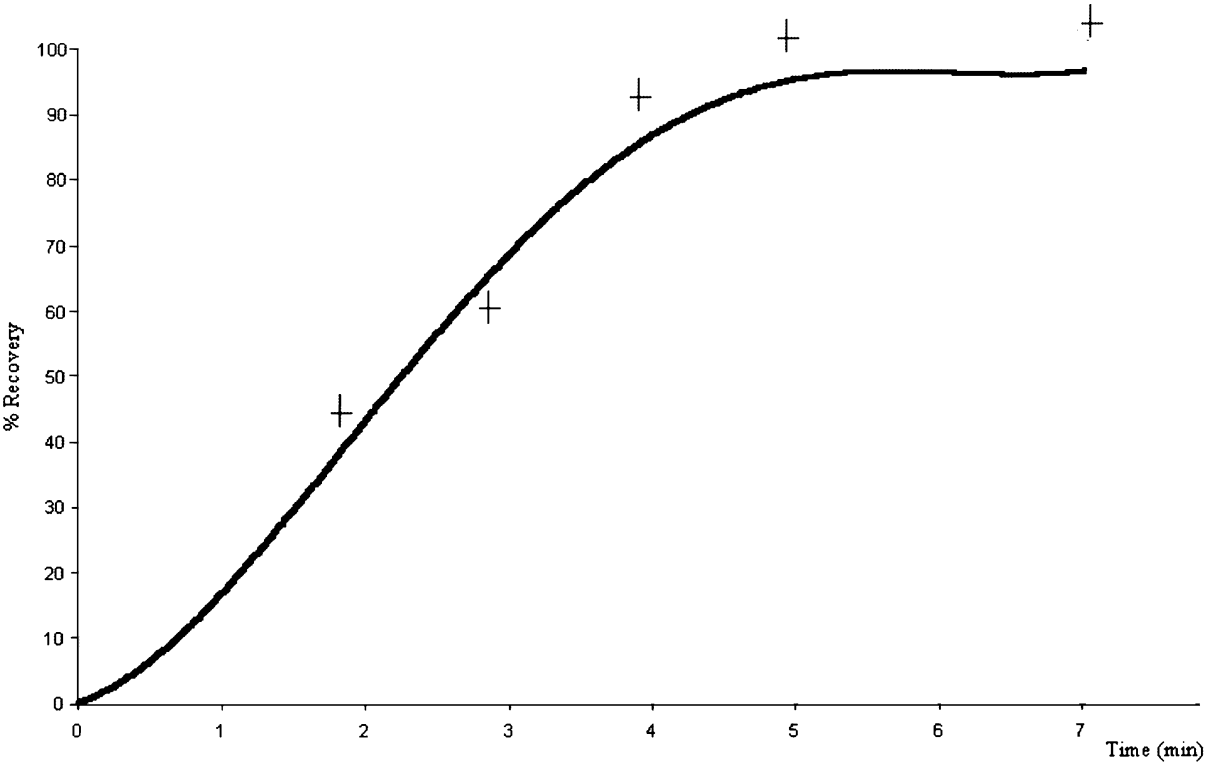


Fig. 6. Influence of the extraction time on the SFE efficiency for fat from foods (milk, cocoa). Amount of sample = 0.01 g; SFE conditions: CO_2 flow-rates = 1 ml min^{-1}; pressure = 256 bar; extraction temperature = 100°C; stainless steel trap; nozzle (restrictor) and trap temperature during collection = 70 and 55°C, respectively; rinse solvent = *n*-hexane; rinse volume = 1 ml; nozzle and trap temperature during rinse = 60 and 45°C, respectively. % recovery of fat matter is expressed as % w/w palmitic acid. 2 Min for the static extraction period.

Table 1
Figures of merit of the proposed method for the determination of fat in foods

Equation[a] ($n=27$)	$S=9.982C-0.215$
Regression coefficient	0.9942
R^2 (from ANOVA test) (%)	98.8
Standard deviation of residuals, $S_{y/x}$	0.0290
Determination range (% w/w)	0.005–0.070
Throughtput (samples h^{-1})	6
Detection limit[b] (% w/w)	0.002

[a] Dependent variable, S=measured signal [frequency difference ΔF (kHz)]; independent variable, C=% w/w fat.
[b] Defined as the blank signal plus three times its standard deviation.

The additional void volume was offset by adding 1 g of diatomaceous earth as an inert solid. An identical amount of diatomaceous earth was extracted under the same conditions in order to confirm that no interferent were extracted from this material.

3.1.5. Collection and elution conditions

Stainless steel balls and the ODS were tested as packing materials for the analyte trap. *n*-Hexane was used as the rinse solvent with both types of packing. *n*-Hexane is an excellent rinse solvent for fats [33], as its non-polar nature facilitates collection of the analyte. The stainless steel ball trap was selected. Its inert packing material proved to be highly efficiency in collecting the fat matter. A volume of 1.0 ml of rinse solvent (*n*-hexane) at 1.5 ml/min and 45°C was large enough to remove the whole fat fraction from the trap. Similar results were obtained with the ODS trap with low concentrations of fat; however, poor results were obtained at high concentrations of fat owing to the loss of analytes.

3.2. On-line SFE with PZ detection

In the on-line extraction mode, the supercritical fluid was decompressed through the restrictor (nozzle) to allow the deposition of the extracted fat fraction at the interface (trap, stainless steel junction and manifold). The expansion of the supercritical fluid is generally accompanied by a positive Joule–Thompson coefficient that results in a cooling effect [6]. However, a single-dimension compressible flow-model [6] predicts the decompression outside the restrictor, and, at the sufficiently high linear velocities obtained, even relatively non-volatile components are expected to travel for some distance prior to deposition. The additional external cooling (cryogenic CO_2) of the SFE extractor in the decompression zone may help to contain the extracted analyte. The trapping conditions used in this work were described in Section 3.1. Other connections of the interface were also found to be critical. In the extraction mode, the fat fraction was deposited in the trap. Gaseous CO_2 was vented to the atmosphere by flowing through the transfer tubing and valve IV. The inner diameter of the transfers tubing and injection-loop were found to play key roles in the collection of fats. With a small tubing (diameter 0.80 mm I.D.), sufficient back-pressure was introduced so decom-

Table 2
Analysis of synthetic samples containing palmitic acid as fat matter

Concentration added (% w/w)	Concentration found (% w/w)	Error (%)
0.0050	0.0046	−8.8
0.0060	0.0057	−5.0
0.0100	0.0095	−5.2
0.0100	0.0097	−3.0
0.0080	0.0089	+5.8
0.0200	0.0210	+4.6
0.0290	0.0290	−2.8
0.0430	0.0420	−1.5
0.0510	0.0500	−1.2
0.0600	0.0590	−1.8
0.0690	0.0700	+1.0
0.0710	0.0700	−2.5

Table 3
Analysis of food samples (milk, cocoa) by using the proposed and Soxhlet methods

Sample	Concentration found (% w/w)	
	Proposed method	Soxhlet method
Skimmed milk (Prika)	1.20±0.01	1.1±0.2
Whole milk (Unknown brand)	25.0±0.5	24.60±0.02
Skimmed milk (Nestlé)	1.19±0.01	1.00±0.04
Cocoa (Nesquick)	3.81±0.04	3.65±0.04
Cocoa (Eroski)	3.72±0.08	3.62±0.04

pression occurred along the tubing to the outlet. Different loop sizes were tested (150, 350 and 500 μl). A 350-μl loop was found to provide the best results (a 150-μl loop presented low detection problem, whereas a 500-μl loop saturated the response of the detector).

3.3. Figures of merit and validation of the proposed method

Standards of palmitic acid (fat fraction) at concentrations between 0.005 and 0.070% w/w were used to construct the calibration graph for the determination of fats. The figures of merit of the proposed method are listed in Table 1. The calibration graph (run from triplicate measurements at each point) was linear over the range 0.005–0.070% w/w. The precision of the method, expressed as relative standard deviation for 0.01% w/w palmitic acid was ±2.3% ($n=11$; $P=0.05$) on different days. The estimated throughput was 6 determinations/h. The proposed method is thus an expeditious alternative to the Soxhlet method (using *n*-hexane with solvent) which takes about 12 h per analysis.

The validation of the proposed method against the Soxhlet method using regression analysis was studied. The results provided by the Soxhlet method were used as the independent variable (*x*-axis) and those obtained with the proposed method as the dependent variable (*y*-axis). Calculations showed standard errors to be insignificant. With 13 degrees of freedom ($n=2$), the following confidence limits ($P=0.05$) were obtained for the slope $b=1.0130\pm0.0287$ and the intercept $a=0.1152\pm0.3252$. Based on these results, the slope and intercept were not significantly different from the ''ideal'' values of 1 and 0, respectively; hence, no systematic differences between both series of results existed. Therefore, the proposed method is traceable to the official method.

3.4. Analysis of synthetic and real samples

The applicability of the proposed method was initially checked by analyzing synthetic samples of fat. The results obtained are shown on Table 2. As can be seen, differences between the concentrations found and those added were in general small (average error was 3.6%). Subsequently, the method was applied to real samples (milk and cocoa of different brands). Amounts of sample of 0.05–0.10 g were accurately weighed and added to 1 g of diatomaceous earth held in the thimble. Matrix effects were cancelled by virtue of the samples being extracted within the SFE system. The results obtained were compared with those provided by the Soxhlet method. As can be seen from Table 3, both were quite consistent, as further confirmed by the results of the *t*-paired comparison test. The null hypothesis was H_0: mean $(d)=0$, versus the alternative one H_1: mean $(d)\neq0$. The computed value for *t* was less than the critical value (with $\nu=14$ degrees of freedom at the 95% confidence level, $t_{crit}=2.145$), so H_0 was not rejected and no differences exist between both methods. Consequently, the proposed method can be applied with satisfactory results to real samples.

4. Conclusion

The results of this work demonstrate that the proposed on-line SFE-PZ system provides all the advantages of a multi-dimensional screening system (high sensitivity, precision, simplified sample hand-

ling and automatability). Samples that typically require extraction for a long time can be analyzed with little sample preparation. The results show that very small amounts of samples can indeed be analyzed with reproducible results. The use of organic solvents is significantly reduced with the on-line approach, which avoids the dangers associated with exposure to and the environmental release of hazardous materials. In addition, the probability of analyte losses and sample contamination is minimized. The proposed approach should also be applicable to the determination of a variety of analytes in complex matrices.

Finally, the piezoelectric detector has high sensitivity and can be used for very broad ranges of compounds. However, its response is not selective.

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