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Improved sample treatment for the determination of insoluble soap in sewage sludge samples by liquid chromatography with fluorescence detection

Samuel Cantarero[a], A. Zafra-Gómez[a,*], O. Ballesteros[a], A. Navalón[a], J.L. Vílchez[a], G. Crovetto[a], C. Verge[b], J.A. de Ferrer[b]

[a] Research Group of Analytical Chemistry and Life Sciences, Department of Analytical Chemistry, University of Granada, Campus of Fuentenueva, E-18071 Granada, Spain
[b] Cepsa Química S.A., Avda. del Partenón 12, E-28042 Madrid, Spain

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ABSTRACT

A new selective and sensitive method for the determination of insoluble fatty acid salts (soap) in sewage sludge samples is proposed. The method involves a clean up of sample with petroleum ether, the conversion of calcium and magnesium insoluble salts into soluble potassium salts, potassium salts extraction with methanol, and a derivatization procedure previous to the liquid chromatography with fluorescence detection (LC-FLD) analysis. Three different extraction techniques (Soxhlet, microwave-assisted extraction and ultrasounds) were compared and microwave-assisted extraction (MAE) was selected as appropriate for our purpose. This allowed to reduce the extraction time and solvent waste (50 mL of methanol in contrast with 250 mL for Soxhlet procedure). The absence of matrix effect was demonstrated with two standards ($C_{13:0}$ and $C_{17:0}$) that are not commercials and neither of them has been detected in sewage sludge samples. Therefore, it was possible to evaluate the matrix effect since both standards have similar environmental behaviour (adsorption and precipitation) to commercial soaps ($C_{10:0}$–$C_{18:0}$). The method was successfully applied to samples from different sources and consequently, with different composition.

1. Introduction

Soap has been used worldwide since ancient times and in particular since Leblanc developed an industrial method to produce caustic soda in 1791 [1]. In the last century, the use of soap has provided countless benefits for the human activity and it development [2].

Fatty acid salts (soap) are defined as the salts of monocarboxylic acids ranging, in number of carbon atoms, from 10 to 22. The $C_{16:0}$–$C_{22:0}$ members of the group may be saturated or unsaturated with a carbon–carbon double bond [3]. Soap is the most widely used anionic surfactant for cleaning products, cosmetics, coatings, lubricants, emulsifier and other industrial applications. Uses in household cleaning products, include fabric washing products, fabric conditioners, laundry additives, and surface and toilet cleaners [4]. The total fatty acid salts estimated for use as household detergents and cleaning products is 71,306 annual tons [3]. The level of soap found in the different washing products is variable and it ranges approximately from 0.1 to 10.5% in regular powder, 2–20% in regular liquid, 0.1–3.4% in compact powder, 4–10% in compact liquid, 0.7–2% in tablets and 13.1–15.1% in compact gels [5]. From a commercial point of view, the most important soaps are $C_{12:0}$ (lauric acid), $C_{14:0}$ (miristic acid), $C_{16:0}$ (palmitic acid), $C_{18:0}$ (estearic acid) and $C_{18:1}$ (oleic acid) predominantly with counterions of sodium and potassium.

As it has been previously mentioned, there is a high human and industrial associated activity with soap and relatively large amounts of these compounds are released into the environment. Wastewater is the most common way to introduce these compounds into the environment either via direct discharge (without treatment) or after degradation in waste water treatments plants (WWTP).

In practice, the most important way for eliminating these products in environment is biodegradation [6–9]. However, while the use of sodium salts are by far the most common use of soap in finished products, the predominance of calcium and magnesium ions in wastewater leads to rapid formation and predominance of relatively insoluble calcium and magnesium salts. Therefore, the rate of biodegradation mainly depends on the physical and chemical characteristics, in particular solubility and adsorption. Taking into account this evidence, the fate of fatty acid salts is strongly influenced by the poor water solubility of the calcium and magnesium salts [10].

From an analytical methodology point of view, in the revised literature, the majority of proposed methodologies are based on official methods [11–14] and they are used in soap industry for the quality control of finished products. There are some works for the

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* Corresponding author. Tel.: +34 958 243326; fax: +34 958 243328.
E-mail address: azafra@ugr.es (A. Zafra-Gómez).

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determination of the total amount of soap by gas and/or liquid chromatography based on the hypothesis that the behaviour of soap in solution is analytically similar to a fatty acid [15–20]. However, there are a limited number of articles concerned to the determination of soap in environmental matrices [21–25]. Prats et al. [21] and Matthijs et al. [22] have carried out two studies in order to monitoring the behaviour of soaps from the discharge to wastewater until the finalization of the treatment in wastewater treatment plants (WWTP).

This lack of methodologies is probably due to the behaviour of soap in the environment. In the presence of calcium or magnesium salts, the soap tends to precipitate as insoluble salts, complicating the determination of the compounds. To solve this analytical problem, the transformation of soap in potassium salts with potassium carbonate in the presence of an excess of tripotassium ethylenediaminetetraacetate previous to analyte extraction is proposed in this work. An improved analytical method for the determination of soap in sewage sludge samples is then proposed. The validation was carried out by spiking dry natural samples with two standards ($C_{13:0}$ and $C_{17:0}$). Neither of them has been detected in sewage sludge samples so it is possible to evaluate the matrix effect since both standards have similar environmental behaviour (adsorption and precipitation) to soap ($C_{10:0}$–$C_{20:0}$). The most usual techniques of extraction (Soxhlet, microwave-assisted extraction (MAE) and ultrasound) have been also compared.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were of analytical grade unless specified otherwise. Commercial fatty acids $C_{10:0}$–$C_{18:1}$ (purity 97–99%) were supplied by Merck (Darmstadt, Germany). Fatty acid potassium and calcium salts were synthesized in our laboratory (purity 98–99%).

Stock solutions of calcium and potassium soap (100 mg L^{-1}) were prepared in isopropanol and methanol, respectively, and stored at 4 °C in the dark. These solutions were stable for at least 6 months. Working standards for potassium soap were prepared just before use by diluting with methanol.

Methanol and acetonitrile (HPLC gradient-grade) used as mobile phase were supplied by Merck (Darmstadt, Germany). 4-Bromomethyl-7-methoxycoumarin (BrMMC), 18-crown-6-ether, potassium carbonate and tripotassium ethylenediaminetetraacetate (EDTA-K_3) were supplied by Sigma–Aldrich (Madrid, Spain). Potassium phosphate dibasic, trifluoroacetic acid and formic acid (PAI grade) assayed as mobile phase modifiers were supplied by Panreac (Barcelona, Spain). Ethyl-acetate, hexane, cyclohexane, methanol, isopropanol, acetone and petroleum ether (PAI grade) used in the extraction and clean up were supplied by Panreac (Barcelona, Spain). Water (18.2 MΩ cm^{-1}) was purified with a Milli-Q plus system (Millipore, Bedford, MA, USA). All solvents and solutions prepared for LC were filtered through 0.22-μm nylon disk filters (Millipore) before use.

2.2. Instrumentation and software

Microwave-assisted extraction system (MARS5 form CEM Corporation, New Jersey, USA); Ultrasonic irradiation model S-450D from Branson (Danbury, CT, USA), equipped with an ultrasonic generator, a model 102 converter, standard titanium disruptor horn of 12.70 mm diameter, flat and replaceable titanium tip of 12.70 mm diameter and temperature probe; and Soxhlet (250 mL capacity) were employed for solid-liquid extractions. A centrifuge, model Universal 32, from Hettich (Tuttlingen, Germany) was used to separate solid and liquid phases. The chromatographic system consisted of an Agilent 1100 series high performance liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump, an online degasser, an autosampler, an automatic injector with a loop of up to 100 μL, a thermostated column compartment and a fluorescence detector (flow cell volume 8 μL) connected online. ChemStation for LC 3D (Agilent) software package was used to control the instrument, data acquisition and data analysis. A Luna C_{18} (150 mm × 4.6 mm i.d., 5 μm particle size) from Phenomenex (Torrance, CA, USA), a Spherisorb ODS-2 (250 mm × 4.6 mm i.d., 5 μm) and an Atlantis dC18 (150 mm × 4.6 mm i.d., 5 μm) both from Waters (Milford, MA, USA), two LiChrospher-100 RP-18 column (250 mm and 125 mm × 4 mm i.d., 5 μm) and two LiChrospher-100 RP-8 column (250 mm and 125 mm × 4 mm i.d., 5 μm) from Merck were assayed. Statgraphics software was used for the statistical analysis of data [26].

2.3. Sample preparation

For application of the method, 25 sewage sludge samples were collected from different WWTP of Spain. For evaluation of matrix effect samples were collected from Estepona WWTP (in the south of Spain). The samples were conserved in plastic flask and immediately adding 3% (v/v) of formaldehyde. Once in the laboratory, samples were dried in a heater at 105 °C until constant weight, and later milled and strained through a 0.25 μm sieve in order to enhance the extractability of the analyte. The samples were kept in the dark at 4 °C until analysis.

Spiked sewage sludge samples were prepared by addition to 5 g of accurately weighted, in stainless steel flasks, sample of suitable volumes of standard solutions of analytes in isopropanol. Then, 25 mL of methanol were added until the solvent completely covered the samples and the suspension was thoroughly mixed for 5 h with a mechanical shaker to enable analytes distribution throughout the solid. The solvent was slowly evaporated at room temperature over a period of 24 h until constant weight and the spiked samples were then ready for the experiments.

2.4. Basic procedure

2.4.1. Microwave

5.0 g of sewage sludge sample were thoroughly weighed in a microwave vessel and suspended into 50 mL of petroleum ether. The suspension was properly covered and extracted during 20 min (10 min for holding) at 70 bars and 1600 W in order to remove interferences (free fatty acids, lipids, etc). The supernatant was then accurately removed and 50 mL of water, 50 mg of EDTA-K_3 and 25 mg of K_2CO_3 were added to the solid fraction. The mixture was heat for 4 h at 105 °C in order to transform soap calcium salts into potassium salts and afterwards, the supernatant was accurately removed and 50 mL of methanol were added for the analyte extraction. The procedure was carried out at 70 bars and 1600 W for 10 min (10 min for holding). After extraction was completed, 1 mL of internal standard solution (40 mg L^{-1}) was added to 2 mL of the methanolic extract and the mixture was evaporated to dryness under a nitrogen stream. For the derivatization procedure, 100 μL of the BrMMC (0.2%, w/v, in acetonitrile) and 100 μL of the 18-crown-6-ether (0.5%, w/v, in acetonitrile) were added and the mixture was heated at 110 °C for 5 min. Finally, the extract was diluted with 3.8 mL of water and filtered through 0.22-μm nylon Millipore filters before injection into the LC system.

2.4.2. Soxhlet

For 5 g of sample, the interference removing procedure was carried out with 250 mL of petroleum ether at 70 °C for 12 h. The supernatant was then accurately removed and the conversion of soap calcium salts into potassium salts was developed with 50 mL of water, 50 mg of EDTA-K_3 and 25 mg of K_2CO_3 at 105 °C for 4 h. Finally, 250 mL of methanol and 6 h at 100 °C were necessaries to extract the soap. The mixture was then centrifuged at 4000 rpm for 10 min. As a consequence of the high volume, concentration (heater 80 °C, 1 h) of the liquid extract in 50 mL was required. After concentration, an aliquot of 2 mL of the concentrate extract was treated using the same procedure as microwave extraction

2.4.3. Ultrasound

The bath was prepared by addition of distilled water (1.5 L). Each phase of the extraction was performed using two metallic capsules within the sonication bath at the same time. For 5 g of sample, the elimination of interferences was carried out with 50 mL of petroleum ether for 20 min and with amplitude of 75%. The supernatant was then accurately removed and the conversion of calcium into potassium salts was developed with 50 mL of water, 50 mg of EDTA-K_3 and 25 mg of K_2CO_3 at 105 °C for 4 h. Finally, the analytes were extracted with 50 mL of methanol for 20 min with amplitude of 75%. The extract was then centrifuged at 4000 rpm for 10 min and an aliquot of 2 mL was treated using the same procedure as microwaves assisted extraction and Soxhlet extraction.

2.5. Liquid chromatography analysis

The analytical chromatographic columns listed in Section 2.2 were tested. The LiChrospher 100 RP-8 (250 mm × 4 mm i.d., 5 μm) coupled with a 15 mm safeguard column gives us the best results in terms of resolution between different analytes, mainly for the critical pair $C_{16:0}$–$C_{18:1}$, time of chromatogram and peak shape.

Different mobile phases were studied in order to optimize the separation. Methanol and acetonitrile were checked as organic solvents and formic acid 0.1% (v/v, pH = 2.4), trifluoroacetic acid 0.1% (v/v, pH = 2.5) and four phosphate buffer solutions (pH 4, 5, 7 and 8) were tested as additives. Acetonitrile gave better results than methanol and we found that the chromatographic parameters were independent on the pH of the mobile phase. Therefore, deionized water (solvent A) and acetonitrile (solvent B) were used. The initial conditions were 30% (v/v) of A, 70% (v/v) of B and flow 1.5 mL min^{-1}. A linear gradient in three steps was then established in order to reach 80% (v/v) of B at 10 min, 85% (v/v) of B at 15 min (held for 5 min) and 100% (v/v) at 25 min. The amount of B was then immediately set at 70% (v/v), remaining stable for 5 min to restore the initial conditions and for column conditioning. The total run time was 30 min and the column oven was set at 30 °C.

The excitation and emission wavelengths for fluorescence measurements were also optimized. The higher fluorescence signal was obtained at 329 nm for excitation and 398 nm for emission, respectively, using an optimized PMT gain of 12.

Lastly, a study to evaluate the possibility of increasing the injection volume was carried out. A range from 10 to 100 μL was studied and no extra broadening of the peaks was observed, even at maximum value. Accordingly, 100 μL was chosen as the injection volume.

3. Results and discussion

3.1. Interference removing

As we remarked in the introduction of this paper, while the use of sodium salts is by far the most common use of soap in finished products, the high levels of calcium and magnesium ions in wastewaters lead to rapid formation of relatively insoluble salts. As well, the precipitated/adsorbed salts are less disposable for microorganisms and biodegradation is notably lower. Therefore, soap is usually present in sewage sludge in the form of precipitated calcium or magnesium salts. However, free fatty acids can be present in a sample together with insoluble soap, and it is not possible to differentiate between those compounds in the extraction and derivatization steps. Sewage sludge is rich in free fatty acids and lipids; therefore, it is necessary to introduce a previous step for the elimination of those interferences. Table 1 shows a comparison of soap concentration in three samples of sewage sludge with and without interference removal step using Soxhlet, ultrasounds and microwaves for extraction of analytes.

Table 1
Comparison of soap concentration with and without interference removal.

	Soxhlet Concentration (g kg^{-1})	Ultrasound Concentration (g kg^{-1})	Microwave Concentration (g kg^{-1})
Without interference removal			
Sewage sludge 1	130.6	127.7	129.7
Sewage sludge 2	56.8	54.3	58.0
Sewage sludge 3	13.9	13.2	13.7
With interference removal			
Sewage sludge 1	23.7	22.1	23.1
Sewage sludge 2	8.2	8.0	8.1
Sewage sludge 3	1.7	1.6	1.6

It can be observed that the interference due to the presence of fatty acids or lipids in the samples is very remarkable and the result without the clean up step is notably higher than the one obtained when the interference removal procedure is applied.

In order to remove interferences, the solvent was selected following the methodology proposed by Moreno et al. [27]. Petroleum ether, hexane, cyclohexane, ethyl-acetate and methanol were assayed. Petroleum ether, ethyl-acetate and methanol removed percentages of fatty acids closed to 100%. However, the last two mentioned solvents also removed a 7 and 12% of the soap respectively. Therefore, petroleum ether was selected for our purpose. Once the solvent was selected, three different extraction methodologies were assayed. Five grams of sample was treated in all cases.

3.1.1. Microwave-assisted solvent extraction

The influence of the solvent volume, the applied microwave power, the irradiation time, the temperature and the pressure during the clean up procedure were investigated. The solvent volume was varied between 10 and 70 mL (maximum solvent amount for microwave vessel). A volume of 50 mL gave the best results. The microwave power was varied between 100 and 1600 W (maximum). The best result for the simultaneous extraction of the maximum number of samples (fourteen) was 1600 W. The temperature in the extraction procedure must be higher than the boiling point of the employed solvent. However, an excessive increase in temperature produces a notable increase in pressure to reach the maximum tolerable (100 bars) and a subsequent leak of solvent and the immediate stop of the instrument are originated. That is the reason why the pressure was optimized and fixed in 70 bars in order to obtain an extraction temperature higher than the solvent boiling point and avoiding the overpressure. Finally, the extraction time was studied between 5 and 25 min. The higher extraction yield (99%) was obtained at 20 min, remaining constant for longer times

3.1.2. Soxhlet

The previously proposed methodology by Moreno et al. [27] was followed. However, the extraction time was modified. The parameter was varied between 6 and 72 h and the higher yield in the fatty acids elimination (99%) was obtained at 12 h

3.1.3. Ultrasound

Each phase of the extraction was performed using two metallic capsules within the sonication bath at the same time. The solvent volume, the extraction time and the ultrasound amplitude were

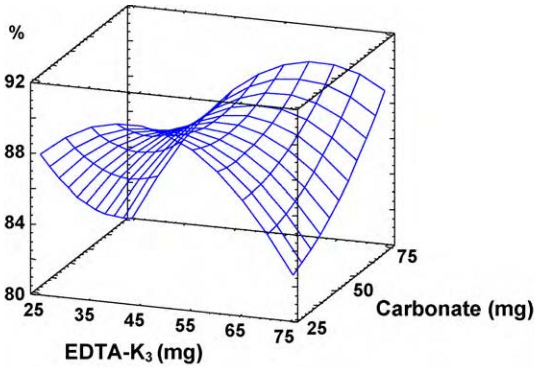

Fig. 1. Conversion of soap into potassium salts. Reagent amounts effect over the reaction efficiency.

studied. The solvent volume was varied between 10 and 70 mL (maximum solvent amount for capsule used) and a volume of 50 mL gave the best results. The extraction time was varied between 5 and 25 min and the amplitude between 25 and 75% (maximum). The time was fixed in 15 min and the amplitude in 75%.

3.2. *Conversion of soap into potassium salts*

Since fatty acid calcium and magnesium salts are practically insoluble in water and most other solvents, when soap appears in sewage sludge in the precipitated calcium o magnesium salts, the extraction procedure and the derivatization with BrMMC are difficult, tedious and not reproducible. In order to avoid those problems, the conversion of soaps into potassium salts proposed by Hayashi et al. [23] was used in this work. Briefly, the procedure consists in a reaction of calcium or magnesium salts with potassium carbonate in the presence of EDTA-K_3 in aqueous media at high temperature.

Because some of the potassium salts are slightly soluble in water, it is important to emphasize the necessity of removing the water used as reaction medium in the conversion procedure to favour the subsequent extraction with the adequate solvent. In fact, we use a reaction temperature of 105 °C (higher than water boiling point) to remove water through the conversion procedure during the time necessary (4 h) to complete the whole transformation. As well, in order to minimize the dry time, we added just the minimum volume of water for the complete homogenization of sample with the reagents (50 mL).

The amounts of tripotassium ethylenediaminetetraacetate and potassium carbonate to be used are related to each other; consequently these variables were optimized using response surface methodology. This was done in order to obtain the maximum response while varying simultaneously these two parameters. We applied an orthogonal cubic face centred experimental design. All calculations were carried out with the Statgraphics data analysis package [26]. The amounts of the reagents were varied between 25.0 and 75.0 mg. Fig. 1 shows the response surface obtained.

The optimum for the sum of the studied homologues is reached when 50 mg of the former and 25 mg of the latter salts were used.

3.3. *Extraction of analytes*

Taking into account the different polarities of the analytes, a wide range of polarities was assayed. Ethyl-acetate, acetone, water and methanol were tested as extracting solvents. To select the best extraction solvent, 5.0 g of sewage sludge spiked to get a final concentration of 50 g kg^{-1} of soap in sample and then extracted with 250 mL of each solvent in Soxhlet for 24 h. The extraction yields of soap total were between 85% for acetone to 92% for methanol. This one was the selected solvent as the most adequate, in order to obtain an adequate signal for all of them.

Different extraction techniques for analyte isolation were used in order to select the most effective process for our research. Microwave-assisted extraction, Soxhlet and ultrasounds procedures were assayed and compared.

3.3.1. *Microwave-assisted solvent extraction*

The recovery of compounds from samples is highly influenced by the solvent volume, applied microwave power, irradiation time, temperature and pressure during the extraction procedure. Therefore, the effect of those variables was investigated. The solvent volume was varied between 10 and 70 mL (maximum solvent amount for microwave vessel) and a volume of 50 mL gave the best results for the investigated compounds. A lower solvent volume led to lower recoveries, probably due to a saturation of the solvent by the extracted compounds (especially material from the sludge). Increasing the solvent did not increase the extraction efficiency. Instead, it led to a more diluted extract so that a longer time was required for concentration of extracts. The microwave power was varied between 100 and 1600 W (maximum). The best result for the simultaneous extraction of the maximum number of samples (fourteen) was 1600 W. Following the same reasoning show in Section 3.1, the pressure was optimized and fixed in 70 bars in order to obtain an extraction temperature higher than the solvent boiling point and avoiding the overpressure

Finally, the extraction time was studied between 5 and 25 min. Fig. 2 shows the evolution of the extraction yield when the time is increased.

The higher extraction yield (90%) was obtained at 10 min; remaining constant for longer times. This value was selected as optimum time for extraction.

3.3.2. *Soxhlet*

The previously proposed methodology by Moreno et al. [27] was followed. The extraction time was also optimized in that case. The parameter was varied between 4 and 48 h and the higher extraction yield (90%) was obtained at 6 h remaining constant for longer times (see Fig. 2).

3.3.3. *Ultrasound*

The solvent volume, the extraction time and the ultrasound amplitude were studied. The solvent volume was varied between 10 and 70 mL (maximum solvent amount for vessel used). A volume of 50 mL was selected.

The extraction time and the amplitude are related to each other; these variables were also optimized by using response surface methodology. We applied an orthogonal cubic face centred experimental design. The extraction time was varied between 5 and 25 min, and the amplitude from 25 to 75% (maximum). Fig. 2 shows the response surface obtained. An extraction time of 10 min and 75% of amplitude were fixed. These values were selected for a compromise between reasonable recovery, reasonable analysis time, and smaller consumption of the horn tip rather than the optimum values found.

3.4. *Derivatization procedure*

The fatty acid potassium salts have not intrinsic fluorescent. Once the extraction was carried out, a derivatization procedure to enhance the fluorescence of analytes was necessary. There is a high amount of derivatization reagents for the determination of fatty acid by gas and liquid chromatography [28,29]. In this paper, a modification of the procedure using BrMMC and 18-crown-6-ether proposed by Hayashi et al. [23] was selected. Firstly, a

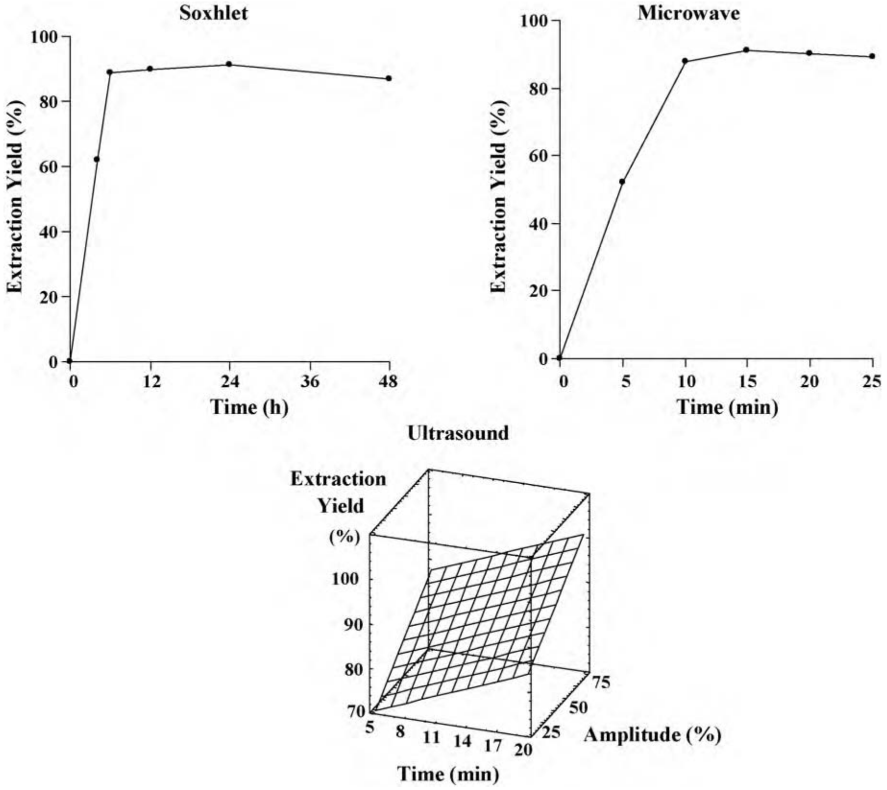

Fig. 2. Extraction of analytes. Influence of the extraction time over the extraction yield.

clear improvement in the analytical characteristics of the chromatographic peaks was observed when acetone was removed from the reaction solvent. The reaction time, the temperature and the amount of reagents were also modified. The effect of the temperature was studied in the range 25–125 °C. The maximal signals were obtained in the range from 95 °C for $C_{8:0}$K to 125 °C for $C_{14:0}$K, $C_{16:0}$K and $C_{18:0}$K. Due to the notable difference between those values, a middle temperature of 110 °C, near to the optimum for all compounds, was selected. The reaction time was also studied between 1 and 90 min at 110 °C and we observed that 5 min was enough to obtain a good reaction yield. Finally, the minimal amount of reagents for the complete reaction of compounds in the range of analyte concentration from 1.0 to 150.0 mg kg^{-1} was studied. BrMMC was assayed at three concentration levels 0.1, 0.2 and 0.3% (w/v) and 18-crown-6-ether at 0.2, 0.5 and 0.75% (w/v). The higher values correspond to the high solubility limits of reagents in acetonitrile. The values selected were 100 μL of each reagent at a concentration of 0.2 and 0.5% (w/v), respectively.

3.5. Comparison of the extraction procedures

Soxhlet, microwave-assisted and ultrasound extraction techniques were compared. A Student's *t*-test was applied to check the statistical similarity between the values of the calibration curves [30] obtained using those procedures. Firstly, it was necessary to compare the variances estimated as $s_{y/x}{}^2$ by means of the *F*-Snedecor test. Table 2 shows the calculated *F*-values and *t*-values for the comparison.

As it can be observed in table, the *t*-values calculated were lower than the theoretical *t*-value in all cases and it can be concluded by H_o, there were no significant differences between the results obtained by the three methodologies. Test performed at significance level of $\alpha < 0.05$. However, it is important to note that the extraction volume is clearly lower for microwave-assisted and ultrasound extraction and the extraction time per sample is clearly lower for MAE related to ultrasounds procedure or Soxhlet. Therefore, this extraction technique was selected for further experiments.

3.6. Evaluation of matrix effect

In the literature, there are some analytical methodologies for the determination of soap in environmental samples using LC or GC as analytical techniques. Despite, nowadays there is not a good methodology capable of taking into account the matrix effect in this

Table 2
Comparison of three extraction methodologies.

	Soxhlet		Ultrasound		Microwave	
	$C_{13:0}$	$C_{17:0}$	$C_{13:0}$	$C_{17:0}$	$C_{13:0}$	$C_{17:0}$
a	20.1	11.2	44.6	17.2	33.1	9.5
s_a	8.7	4.7	7.7	3.7	7.8	1.1
b (kg mg^{-1})	89.2	37.2	87.2	35.9	89.5	36.3
s_b (kg mg^{-1})	2.3	1.6	1.9	2.6	1.3	2.1
$s_{y/x}$	18.0	11.5	5.4	7.0	11.4	4.5

	Statistical comparison of calibration curves					
	SOX *vs* US		SOX *vs* MAE		MAE *vs* US	
	$C_{13:0}$	$C_{17:0}$	$C_{13:0}$	$C_{17:0}$	$C_{13:0}$	$C_{17:0}$
F_{cal}[a]	11.1	2.7	2.5	6.5	4.5	2.4
t_{cal}[b] b	0.7	0.4	0.1	0.3	1.0	0.1
t_{cal}[b] a	2.0	1.0	1.1	0.3	1.0	2.0

a, intercept; s_a, intercept standard deviation; b, slope; s_b, slope standard deviation; $s_{y/x}$, regression standard deviation.

[a] $F_{tabulated}$ (*df*: 17, $P = 0.95$) = 2.34.

[b] $t_{tabulated}$ (*df*: 17, $P = 0.95$) = 2.12.

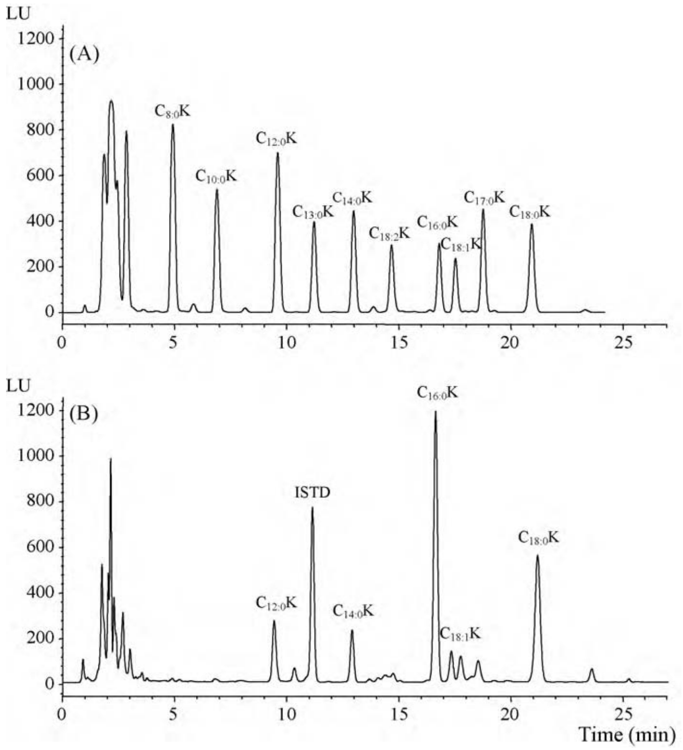

Fig. 3. Representative chromatographic separation of fatty acid salts (soap): (A) standard mixture (5 mg kg^{-1}) and (B) natural (non-spiked) sewage sludge sample.

type of samples. Soap is always present (in higher or lower concentration) in any representative environmental sample. An example of a LC chromatogram of a natural (none spiked) sewage sludge sample is shown in Fig. 3.

In general, for validation, the majority of methods proposed in literature are based in a previous sample treatment to remove analyte contamination before spiking with pure compounds for recovery assays. However, the modification of physical and/or chemical properties of the sample could cause an important change in the capability for soap adsorption and distorting the matrix effect in samples.

In this work, in order to resolve the problem above described, the use of standards with similar chain and analogous environmental behaviour to soap is proposed. A standard with a lower chain ($C_{13:0}$) with similar physical and chemical properties to $C_{10:0}$, $C_{12:0}$ and $C_{14:0}$ and other with a higher chain ($C_{17:0}$) with similar properties to $C_{16:0}$ and $C_{18:0}$ were assayed because they are not commercials and never are presents in the environment.

Firstly, in order to check the presence/absence of matrix effect for $C_{13:0}$ and/or $C_{17:0}$ standard and consequently for soap, a comparison between the calibration curves obtained for six concentration levels in distilled water and the ones obtained, also for six concentration levels, in a natural sewage sludge, adding the compounds before extraction, was developed. Table 3 shows the calibration parameters obtained.

A *t*-Student test was again applied to check the statistical similarity between the values of the slope and intercept for each calibration curve [30] and the calculated *t*-values obtained were lower than the theoretical *t*-value and so show no significant differences in the slopes and intercepts. As the response for both standards was statistically equal, in deionised water and in sewage sludge, the absence of matrix effect was demonstrated. Therefore, by extension, the soap homologues should not present matrix effect.

Table 3
Matrix effect. Comparison of calibration curves in deionized water (standard) and sewage sludge using microwave-assisted extraction.

	$C_{13:0}$		$C_{17:0}$	
	Standard	Sewage sludge	Standard	Sewage sludge
a	47.1	33.6	10.5	11.5
s_a	8.1	12.1	2.1	5.1
b (kg mg^{-1})	93.8	88.1	35.1	39.2
s_b (kg mg^{-1})	3.1	1.7	2.1	2.5
R^2 (%)	99.9	99.6	99.6	99.9
$s_{y/x}$	30.3	25.4	15.1	10.7

	Statistical comparison	
	$C_{13:0}$	$C_{17:0}$
F_{calc}	1.4	2.0
t_{calc} (b)	1.6	1.3
t_{calc} (a)	0.9	0.2

a, intercept; s_a, intercept standard deviation; b, slope; s_b, slope standard deviation; R^2, determination coefficient; $s_{y/x}$, regression standard deviation. F_{tabulado} (*df*: 17, $P = 0.95$) = 2.34; t_{tabulado} (*df*: 17, $P = 0.95$) = 2.12.

3.7. Analytical characteristics of the method

Once the absence of matrix effect was demonstrated, a six point's calibration curve for the LC-FLD method was built by injecting 100 μL of different standard solutions at concentrations of soap total between 1.0 and 150.0 mg kg^{-1} (concentration of soap equivalent in 5.0 g of sample). Each level was prepared by triplicate, and each calibration sample was analysed twice. The standard $C_{13:0}$ was used as internal standard owing to lower analysis time (10 mg kg^{-1}). A calibration curve for each fatty acid salt was constructed using analyte/internal standard peak-area ratio *vs* concentration of analyte. Analytical performance was established according to the Analytical Methods Committee [31], the *lack-of-fit* test was also applied. The results for the intercept (a), slope (b), correlation coefficient (R^2) and probability level of the *lack-of-fit* test, P_{lof} (%), are summarized in Table 4. Thus, the data yield shows good linearity within the stated ranges.

The precision and accuracy data for the analytical procedure are shown in Table 4. Intra-day and inter-day precision (%, RSD) of the method were lower than 5% and were within the acceptable limits to meet the guidelines for analytical method validation which is considered to be ≤20%. The accuracy of the method was also good with the deviation between the nominal concentration and calculated concentration. Precision and accuracy data indicated that the methodology to extract the compounds is highly reproducible and robust.

The detection limit (DL) and quantification limit (QL) were calculated from the blank standard deviation. The chromatographic blanks have been estimated by applying the methodology proposed by González-Casado et al. [32]. It can be assumed that the chromatographic peak shape is a Gaussian-type one, then the estimation of base width (W_b) for 99.73% of the peak-area is $W_b = 6\sigma = 2.548\,W_{0.5\,\text{h}}$, where $W_{0.5\,\text{h}}$ is the half-width of the peak. Extrapolation of the graph of $W_{0.5\,\text{h}}$ at different concentrations of analyte can give us an adequate statistically significant idea of the width of the base for "zero concentration". The blank signal for each analyte can be determined by integration over the baseline of the chromatograms taking a width $t_R \pm 0.5W_{b0}$ where t_R is the retention time of the analyte and W_{b0} has been evaluated as explained above. It relies on studying the blank standard deviation in a time interval corresponding to the peak width at its base, extrapolated to zero concentration. Detection limits which are better adjusted to a statistical evaluation are implemented. Thus, DL and QL were calculated and the results obtained are also summarized in Table 4.

Table 4
Analytical and statistical parameters.

	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:1}$	$C_{18:0}$
a	0.10	0.33	0.43	0.04	0.43	0.13	0.21
s_a	1.12	1.89	0.44	2.43	4.67	5.89	1.34
b (kg mg^{-1})	1.31	1.82	1.78	1.09	1.22	0.41	1.29
s_b (kg mg^{-1})	2.33	2.78	3.56	1.54	2.34	5.67	2.86
R^2 (%)	99.87	99.91	99.93	99.88	99.95	99.96	99.85
LDR (mg kg^{-1})	0.5–150.0	0.4–150.0	0.4–150.0	0.5–150.0	0.6–150.0	0.4–150.0	0.4–150.0
$s_{y/x}$	2.78	4.32	3.36	3.78	3.67	0.76	3.24
P_{lof} (%)	34.5	45.8	67.4	39.7	86.8	57.4	23.7
RSD (%)	4.7	2. 8	2.8	2.6	0.8	1.4	3.0
s_o	0.051	0.044	0.038	0.054	0.058	0.044	0.040
DL (mg kg^{-1})	0.2	0.1	0.1	0.2	0.2	0.1	0.1
QL (mg kg^{-1})	0.5	0.4	0.4	0.5	0.6	0.4	0.4

a, intercept; s_a, intercept standard deviation; b, slope; s_b, slope standard deviation; R^2, determination coefficient; LDR, linear dynamic range; $s_{y/x}$, regression standard deviation; P_{lof}, P value for lack-of-fit test; RSD, relative standard deviation; s_o, blank standard deviation; DL, detection limit; QL, Limit of quantification.

3.8. Application to sewage sludge samples

The proposed method was applied to the determination of insoluble/precipitated fatty acid salts (soap) amount present in 25 sewage sludge samples picked up from different regions of Spain. The microwaves assisted extraction technique was used. A representative LC chromatogram of a natural non-spiked sewage sludge sample is shown in Fig. 3.

The concentration values for six replicate samples are shown in Table 5. The concentration of total soap was determined by interpolation in its standard calibration curve within their linear dynamic range after adequate dilution. The quantification of soap total was achieved assuming that the value found for soap total was the sum of its respective fatty acid salts.

Finally, the percentage average of each individual salt in the natural samples was also determined. The most abundant component is $C_{16:0}$ (47.7%) followed by $C_{18:0}$ (31.1%) and $C_{12:0}$ (10.2%). $C_{14:0}$ (6.8%) and $C_{18:1}$ (4.2%) represent percentages lower than 10% and $C_{10:0}$ was not found in natural samples.

Table 5
Application of the method. Sum of concentrations of homologues $C_{12:0}$–$C_{18:1}$ (microwave).

Spanish region	Province	Concentration[a] (g kg^{-1})	RSD (%)
Andalucía	Sevilla	7.3	2.3
	Sevilla	8.0	1.9
	Sevilla	2.5	2.9
	Cádiz	9.9	3.0
Aragón	Zaragoza	83.3	3.3
Asturias	Oviedo	46.6	3.5
Baleares	Palma de Mallorca	9.7	3.7
	Palma de Mallorca	9.5	3. 9
Cataluña	Barcelona	46.3	3.1
	Barcelona	11.9	3.1
Canarias	Tenerife	6.5	3.8
Castilla-La Mancha	Ciudad Real	7.5	2. 6
Castilla-León	León	17.6	2.7
	León	12.3	2. 9
Extremadura	Badajoz	5.3	2.1
	Mérida	13.4	2.9
Galicia	Pontevedra	27.7	2.0
La Rioja	Logroño	10.9	3.7
Madrid	Madrid	15.5	3.1
	Madrid	8.7	3.1
	Madrid	13.4	3.6
Navarra	Pamplona	74.8	3.3
País Vasco	Guipúzcoa	12.0	3.7
Valencia	Alicante	190.4	3.3
	Alicante	11.5	3.5

[a] Mean value of 6 determinations.

4. Conclusions

An analytical protocol for the determination total soap and in sewage sludge samples based in the isolation of analytes using three different extraction techniques (Soxhlet, microwaves and ultrasound) and a derivatization step, followed by liquid chromatographic with fluorescence detection analysis is proposed. It has been demonstrated that similar results are obtained using different extraction techniques. Microwave-assisted technique presented lower cost in terms of time and amount of solvents. Therefore, it was selected as appropriate for our purpose.

The method has been successfully applied to samples from different sources and consequently, with different compositions. The appropriate sample collection and conservation in conjunction with a previous centrifugation allow good results in comparison with traditional extraction methods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.07.044.

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