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ORIGINAL PAPER

Testing organic solvents for the extraction from fish of sulfophenylcarboxylic acids, prior to determination by liquid chromatography-mass spectrometry

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Abstract The present paper describes the use of different solvent mixtures to extract from fish various sulfophenylcarboxylic acids (SPCs of C_6 to C_{13}), and their originating compounds, linear alkylbenzene sulfonates (LAS of C_{10} to C_{13}). The analytical method utilized involves pressurized liquid extraction, followed by preconcentration of the samples, purification by solid-phase extraction, and finally identification and quantification of the target compounds by high-performance liquid chromatography-mass spectrometry using a system equipped with an electrospray interface operating in negative ion mode. The SPCs and LAS were extracted from spiked fish first with hexane to remove interference from fats, then with different mixtures of solvents: dichloromethane followed by methanol; 50:50 dichloromethane-methanol; and 30:70 dichloromethane-methanol. The LAS recoveries obtained with these three extraction options were high (between 68.5 and 80.8%); however, owing to the low percentages obtained for SPC homologues (13.5, 13.1, and 15.9%, respectively), another extraction procedure with methanol was developed in order to increase these recoveries. The percentage of recovery for total SPCs with the methanolic extraction was higher (90.1%), with a standard deviation of 9.9, and the LAS recoveries also increased (99.9%). Detection limits were between 1 and 22 ng g^{-1} for LAS, and between 1 and 58 ng g^{-1} for SPCs. Quantitation limits were between 4 and 73 ng g^{-1} for LAS, and between 2 and 193 ng g^{-1} for SPCs. This method has been applied to measure the biotransformation of $2ØC_{10}$ LAS (where Ø is a sulfophenyl group) in fish exposed in a flow-through system, and enabled the separation and identification of SPCs from $5ØC_6$ to $9ØC_{10}$.

Keywords Linear alkylbenzene sulfonates · Sulfophenylcarboxylic acids · Mass spectrometry · Environmental analysis · Fish

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Introduction

Millions of tons of surfactants are used every year, mostly in the form of household detergents. Among the many varieties of surfactants, linear alkylbenzene sulfonates (LAS) are the anionic surfactants most commonly used as cleaning agents for laundry purposes. The commercial product is a mixture of homologues, most of them with chain lengths of between ten and 13 carbons atoms (Fig. 1a) and a varying number of positional isomers differing in their location on the aromatic ring. Although their degradation in wastewater treatment plants is very high overall through aerobic processes [1], the presence of LAS in the marine environment is a fact [2–4] and the organisms living in these areas are susceptible to suffering from LAS bioconcentration. The biotransformation of LAS into sulfophenylcarboxylic acids (SPCs) (Fig. 1b) by the organisms is a process that significantly reduces the bioaccumulation potential of LAS, and consists of the ω-oxidation of the alkyl terminus to a carboxylic acid, followed by shortening of the alkyl chain by α-oxidation and β-oxidation (loss of one or two carbons atoms, respectively) [5]. This process generates large quantities of intermediate compounds differing not only in the length of the alkyl chain but also in the link between the sulfophenyl (Ø) group and the alkyl chain [6, 7].

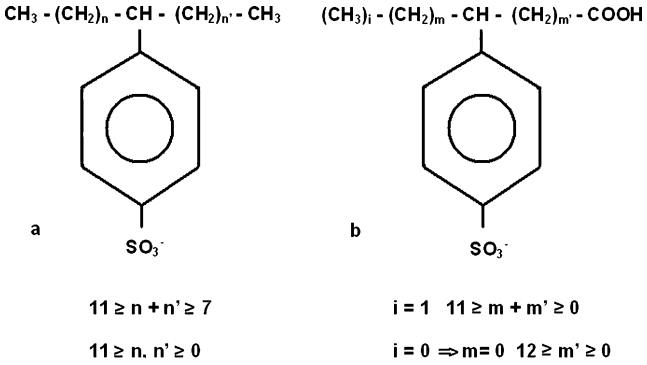

Fig. 1 General chemical structures of linear alkylbenzene sulfonates (LAS) (**a**) and sulfophenylcarboxylic acids (SPCs) (**b**)

Several methods for determining SPCs in different matrixes, such as sewage, sediment, river, marine, estuarine and interstitial waters, have been published [8–12], but in respect of SPCs in organisms, only two techniques have been reported and only one specific SPC homologue was determined by each technique. In 1999 Tolls et al. [13] developed a method for extracting $2ØC_{12}$ LAS together with $3ØC_4$ SPC from freshwater fish. The extraction was performed with matrix solid-phase dispersion (MSPD), followed by the use of graphitized carbon black cartridges, and the detection was performed by high-performance liquid chromatography (HPLC)-fluorescence analysis. The recovery rates of $2ØC_{12}$ LAS and $3ØC_4$ SPC were 84±6 and 65±11% respectively. In the following year, Sáez et al. [14] reported a method based on Soxhlet extraction and solid-phase extraction (SPE) from various marine organisms, and the identification and quantification were performed by HPLC-fluorescence analysis. The selected compounds were C_{11} LAS and C_{11} SPC and the recoveries varied in the range from 80 to 104%. In each published method different extraction techniques, MSPD and Soxhlet extraction, respectively, were used and the final detection and quantification were performed using the same analytical technique: HPLC-fluorescence analysis. Recently, another extraction technique, pressurized liquid extraction (PLE), was successfully applied to extraction of LAS from fish [15], with considerable advantages: PLE reduces consumption of solvent and the time of analysis, eliminates one cleaning stage with SAX minicolumns, and gives a more efficient analysis. This extraction technique has not yet been applied for the extraction of SPCs from organisms. Regarding the fluorescence technique, although this is one of the most frequently applied techniques for the analysis of LAS and SPCs, the compounds are identified by their retention times and, therefore, we would need a standard for each one of the wide variety of SPCs produced by organisms in order to identify them, and not all these standards are available. The liquid chromatography (LC)-electrospray ionization (ESI) mass spectrometry (MS) technique offers the great advantage of distinguishing between SPCs by their specific and quasimolecular $[M-H]^-$ ion fragments, thus allowing their clear identification. Recently, a novel LC-MS/MS method was developed for the determination from feral fish of LAS and its coproducts: dialkyltetralinsulfonates and monomethyl-branched LAS [16]; this technique offers higher sensitivity in comparison with LC-fluorescence analysis.

Recently, intermediate degradation products have been detected in marine organisms during a flow-through experiment [6, 7]; therefore, it is considered necessary to develop a specific analytical method for determining a wide variety of SPC homologues simultaneously with LAS in organisms. To the best of our knowledge, no previous studies of such a method have been reported.

The objectives of this research are:

1. To test different organic solvents for optimizing the simultaneous extraction of LAS (C_{10} to C_{13}) and SPCs (C_6 to C_{13}) from marine organisms using PLE.
2. To identify and quantify SPCs (C_6 to C_{13}) by means of LC-MS.
3. To apply this method to the analysis of samples from fish exposed in a flow-through system.

Experimental

Chemicals

Hexane was purchased from Merck, Darmstadt, Germany; HPLC-grade methanol (MeOH), dichloromethane (DCM), acetonitrile, triethylamine, and acetone were from Scharlau, Barcelona, Spain. Orthophosphoric acid, anhydrous sodium sulfate, sodium chloride, potassium dihydrogenphosphate, acetic acid, and formaldehyde were purchased from Panreac, Barcelona, Spain. The C_{18} minicolumns (500 mg, 6 mL) were supplied by Varian, Harbor City, CA, USA. The D28 filters were purchased from Dionex.

Petroquímica Española supplied the commercial LAS mixture with the following homologue distribution: C_{10} (10.9%), C_{11} (35.3%), C_{12} (30.4%), C_{13} (21.2%). A complete set of SPC standards from C_5 to C_{13} SPC (99% pure) is available, some having been donated and the rest were synthesised at the University of Cádiz.

Sample preparation

Solea senegalensis was a gift from the Marine Culture Wet Laboratory Facilities of Cádiz University with a weight between 5 and 10 g per organism and a total number of 100 fish. The sample preparation was performed following the method used by Álvarez-Muñoz et al. [15]. Briefly, the

organisms were homogenized and the paste obtained was divided into samples of 200-g wet mass each, which were then spiked with commercial LAS and SPC homologues at 5 μg g^{-1}. In order to evaluate the possible original presence of LAS and SPCs, one sample was not spiked and was treated in the same way as the rest. The background levels of the compounds were measured with this sample and they were under the detection limit. For all the samples 4% formaldehyde (8 mL) was added to preserve them. The period of contact between wet mass of the organism and the surfactant was 25 h under continuous agitation, exceeding the time necessary to achieve the equilibrium of adsorption [18]. The samples were frozen and stored at −20 °C until analysis, after being lyophilized and ground in a zirconium oxide ball mill (Retsch PM 200).

Pressurized liquid extraction and solid-phase extraction

Surfactants were extracted from the organisms in triplicate using PLE by means of an ASE 200 accelerated solvent extraction unit from Dionex. The extraction cells (22 mL) were prepared by inserting a disposable cellulose filter into the cell outlet, followed by the sample (about 1 g) and using anhydrous sodium sulfate to improve cell packing. This technique was performed using a first extraction with hexane to remove interference due to the high quantities of lipophilic substances contained in the fish. This extract was not analyzed because neither LAS nor SPCs were detected in this fraction in the previous study by Sáez et al. [14]. Next, a new extraction was performed to test different mixtures of organic solvents by varying their polarity:

Option 1 DCM and MeOH (one extraction with DCM, followed by another with MeOH)
Option 2 DCM-MeOH (one extraction with a 50:50 mixture)
Option 3 DCM-MeOH (one extraction with a 30:70 mixture)
Option 4 extraction with MeOH

DCM was selected as the extraction solvent together with MeOH because this mixture of organic solvents has previously been applied to measure a wide variety of surfactants in marine water and sediments with satisfactory results [12].

The accelerated solvent extraction conditions were as follows: pressure 1,500 psi, temperature 100 °C, static time 5 min, static cycles 3. For option 1, after the extraction with hexane, one extraction with DCM followed by another with MeOH were performed and the two extracts were mixed. For options 2 and 3 the extractions were performed with the corresponding mixture of solvents. The extract volumes were 30 mL; the samples were evaporated to dryness in a rotavapor, and the dry residues were redissolved with 75 mL of warm Milli-Q water in an ultrasonic bath. The SPE was performed using an Autotrace SPE workstation (Zymark) according to the method of León et al. [9]. Sodium chloride (22 g) and potassium dihydrogenphosphate (5 g) were added to the samples and the pH was decreased to 2 with drops of orthophosphoric acid. The C_{18} cartridges were conditioned with 10 mL MeOH and 5 mL Milli Q water and afterwards the samples were passed through them. Then, they were washed with 5 mL water and eluted. The elution was performed with 10 mL MeOH. The eluate was evaporated to dryness and redissolved in 1 mL MeOH-water (80:20) solution containing 1 mg L^{-1} C_{16}LAS as an internal standard.

Liquid chromatography-mass spectrometry

The HPLC system consisted of a Spectrasystem liquid chromatograph with an autosampler, with the injection volume was set at 20 μL. The chromatography separation was done using a reversed-phase C_{18} analytical column (LiChrospher 100 RP-18) of 250 mm×2 mm and 3-μm particle diameter, from Merck. The detection was carried out using an LCQ ion-trap mass spectrometer (Thermo), equipped with an atmospheric pressure ionization source with an electrospray interface. All extracts were analyzed using ESI full-scan negative ion mode in order to determine LAS and SPCs, scanning the mass-to-charge (*m*/*z*) range between 80 and 800. The following mobile phase was used: solvent A 80:20 acetonitrile-water; solvent B water with 5mM acetic acid and 5 mM triethylamine. The elution gradient utilized is described in González-Mazo et al. [19]. The flow rate was 0.15 mL min^{-1}. Other MS parameters were as follows: ion fragmentation energy 40 V, needle tip voltage 4.5 kV, gas stealth flow 60 mL min^{-1}, and ion source temperature 220 °C. The identification of each homologue of LAS and SPCs was carried out by monitoring their quasimolecular ions $[M\text{-}H]^-$ (Table 1) and their specific fragment at *m*/*z* 183. The concentrations of the selected compounds were determined by measuring the peak areas of the quasimolecular ions using external standard solutions (0.5–10 mg L^{-1}) of LAS and SPCs in 80:20 MeOH-water and C_{16} LAS as an internal standard (1 mg L^{-1}).

Clean fish extracts and an 80:20 MeOH-water solution were spiked with 1mg L^{-1} LAS and SPC standards to check the influence of ion suppression (suppression of the analyte signals caused by high concentration of matrix compounds) on the MS detection of the target compounds.

Flow-through experiment

The proposed method was applied to fish samples arriving from a 2ØC_{10} LAS experiment conducted in a flow-through

Table 1 Mass-to-charge (*m/z*) ratios expressed as quasimolecular ions $[M-H]^-$ scanned for the identification of homologues of linear alkylbenzene sulfonates (*LAS*) and sulfophenylcarboxylic acids (*SPCs*)

Homologue	*m/z*
C_{10} LAS	297
C_{11} LAS	311
C_{12} LAS	325
C_{13} LAS	339
C_5 SPC	257
C_6 SPC	271
C_7 SPC	285
C_8 SPC	299
C_9 SPC	313
C_{10} SPC	327
C_{11} SPC	341
C_{12} SPC	355
C_{13} SPC	369

exposure system. The exposure concentration used was 782± 34 μg L^{-1} and the experiment consisted of an exposure phase of 120-h duration followed by a depuration stage of 72 h.

Results and discussion

Extraction efficiency

The results obtained for the total LAS and SPCs together with the recovery percentages of SPC homologues in the spiked fish are shown in Table 2. The recoveries obtained for the three first options tested for total SPCs were in a low range from 13.1 to 15.9%. The results with extraction options 1 and 2 were similar, showing that the extraction efficiency is the same by employing two sequential extractions, with DCM and MeOH, as using only one with a mixture of 50% of the two solvents (in which case less time and less solvent are needed for the extraction). The SPCs have a high polarity and in order to obtain a higher extraction it is necessary to use a more polar organic solvent. Therefore, another option of extraction with MeOH was performed (option 4). The result for total SPCs with MeOH was 90.1% recovery with a standard deviation of 9.9, which demonstrated the high efficiency of PLE in extracting these compounds when the most suitable solvent is employed.

Table 2 shows the SPC homologues identified and quantified in this research and the rates of recoveries obtained. For extractions with options 1, 2, and 3, a slight tendency towards an increased efficiency in the extraction is observed the longer the alkyl chain of the SPC homologues. This is due to the higher hydrophobicty of the longer SPC homologues, their extraction being more efficient when the solvents are less polar, as happens when DCM is mixed with MeOH. With extraction option 4, the recovery percentages were very high for all the homologues, ranging between 83.4 and 114.3%, with standard deviation from 1.6 to 13.1. We also can see that when we used less polar solvents, as in options 1 and 2, the shorter SPC homologues (C_5 and C_6) were not detected, but with more polar solvents, as in options 3 and 4, the C_6 SPC was detected, and only the C_5 SPC could not be identified and quantified.

Comparing the result obtained in this research with the C_{11} SPC previously determined by Sáez et al. [14], we obtained a recovery of 99.1±9.2% by using PLE, similar to the 100±2% calculated with Soxhlet extraction, but more time and more solvent were needed for the analysis

For the total LAS the rate of recovery also increases with the polarity of the solvent mixture utilized, ranging from

Table 2 Recoveries (as a percentage) and standard deviation (n=3) for total LAS, total SPCs and SPC homologues obtained from *Solea senegalensis* (spiked at 5 μg g^{-1}wet mass) with the different extraction options tested

Compound	Recovery (%) ± standard deviation			
	Option 1. DCM and MeOH	Option 2. DCM-MeOH (50:50)	Option 3. DCM-MeOH (30:70)	Option 4. MeOH
SPC_{Total}	13.5±3.3	13.1±0.4	15.9±2.4	90.1±9.9
C_5 SPC	<10.0	<10.0	<10.0	<10.0
C_6 SPC	<10.0	<10.0	16.8±7.6	99.6±10.6
C_7 SPC	13.6±4.6	14.3±2.1	22.1±9.4	92.7±11.5
C_8 SPC	23.6±2.5	21.7±12.9	23.5±5.5	114.3±10.4
C_9 SPC	13.4±6.2	10.7±3.1	<10.0	112.6±5.0
C_{10} SPC	13.1±10.7	16.3±0.2	14.8±2.0	88.4±13.0
C_{11} SPC	<10.0	<10.0	<10.0	99.1±9.2
C_{12} SPC	36±7.3	24.7±0.3	21.1±2.9	97.3±13.1
C_{13} SPC	28.9±2.7	23.3±1.3	21.6±2.2	83.4±1.6
LAS_{Total}	74.2±6.6	68.5±0.8	80.8±8.6	99.9±3.8

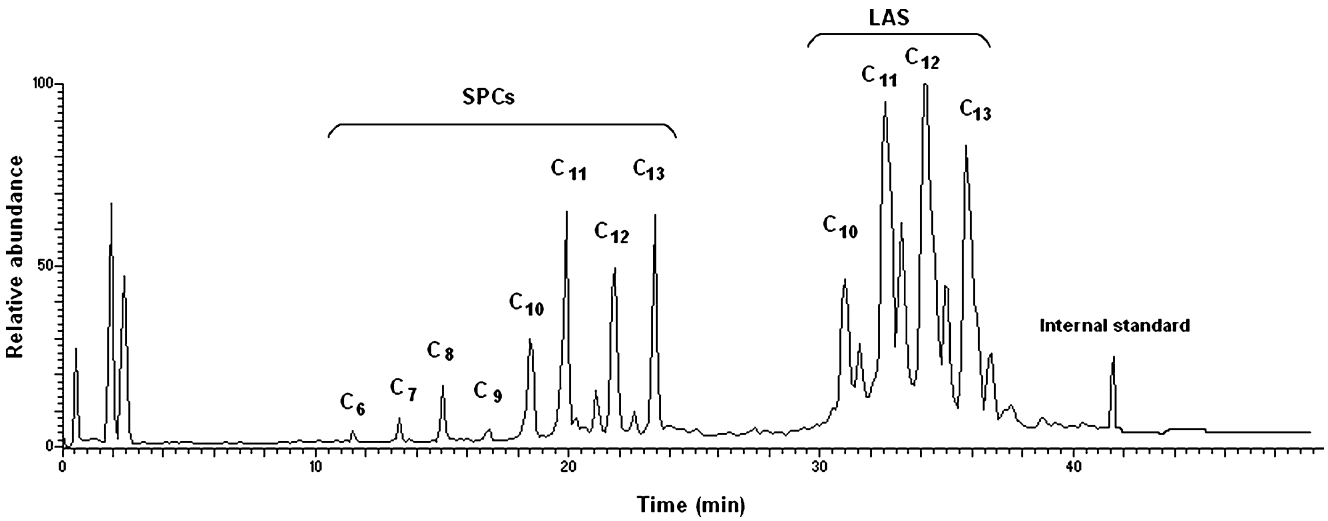

Fig. 2 Full-scan liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS) negative ion mode chromatogram corresponding to a standard LAS plus SPCs

68.5 to 99.9%, with a standard deviation between 0.8 and 8.6. The most efficient extraction solvent was MeOH, in accordance with the results obtained in previous studies [14, 15].

Calibration graphs and limits of detection

The calibration was performed for each LAS and SPC homologue and the relationship between the chromatography response and the concentration was found to be linear for all the target compounds, with coefficients of correlation above 0.9. The limits of detection were calculated at a signal-to-noise ratio of 3:1, and were between 1 and 22 ng g^{-1} for LAS, and between 1 and 58 ng g^{-1} for SPCs. The limits of quantitation were also calculated as the concentration required to increase the signal to 10 times the noise of the baseline, and were between 4 and 73 ng g^{-1} for LAS, and between 2 and 193 ng g^{-1} for SPCs. Both limits were calculated from the spiked samples. The influence of ion suppression was determined to range between 8 and 15% of the signal intensity for each analyte.

Separation, identification, and application

Figure 2 shows a full-scan chromatogram resulting from applying the method described above to a mixture of LAS and SPC standards. The separation of the target compounds in the standards was successful; therefore, the gradient regime used in LC-ESI-MS is appropriate.

The LC-fluorescence analysis technique has been previously applied to determine only one SPC homologue, as we

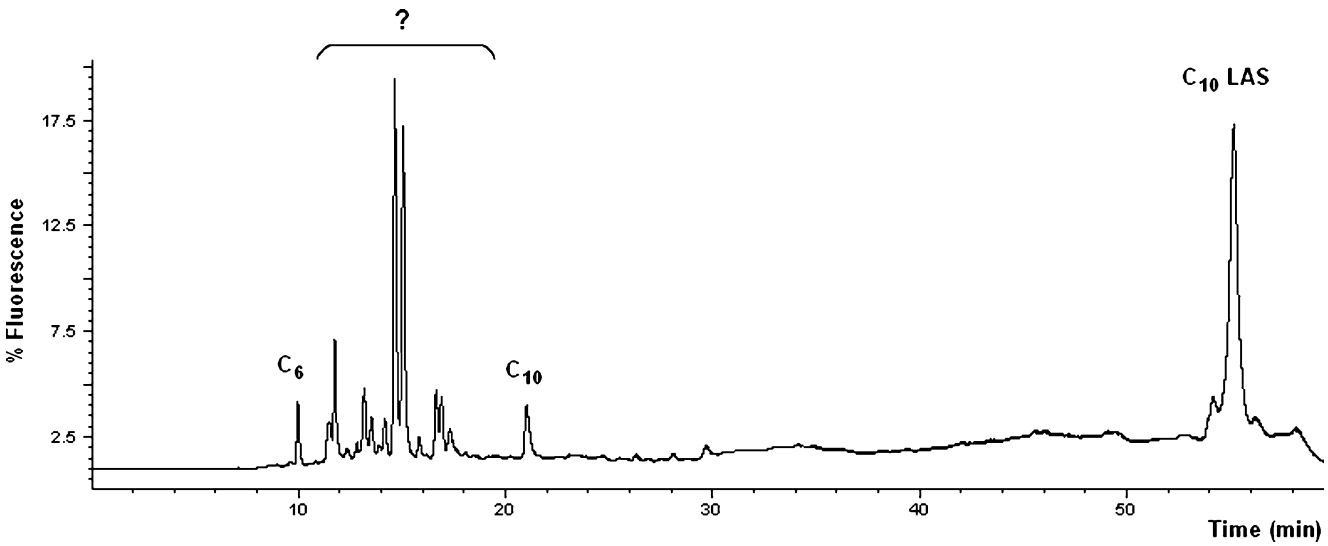

Fig. 3 High-performance liquid chromatograpy-fluorescence chromatogram from an extract of *Solea senegalensis* exposed to 2ØC_{10} LAS (where Ø is a sulfophenyl group)

reported above [13, 14], with good results, but if all the SPCs generated during the biotransformation process are to be identified and quantified, problems appear when a determination is performed with fish samples exposed in a flow-through experiment. During the flow-through experiment the organisms produce a wide variety of SPC homologues and isomers as a result of the biotransformation process. LC-fluorescence analysis allowed us to distinguish some of the homologues, like C_6 and C_{10} SPCs, by their retention times (Fig. 3) by comparing them with the standards, but between them several other peaks appear and their identification is difficult because standards are not available. However, the LC-MS technique allows us to distinguish SPC homologues from their specific quasimolecular ions $[M\text{-}H]^-$ (Table 1), and their specific fragment ion *m/z* 183. Figure 4 presents the chromatograms and the mass spectra from *Solea senegalensis* exposed in a flow-through system at the end of the exposure phase (scanning their quasimolecular ions), showing an unequivocal identification of SPC homologues, which was not possible with the LC-fluorescence analysis technique, and a simultaneous LAS determination. This experiment was performed with the pure homologue $2ØC_{10}$ LAS with an exposure concentration of 782±34 μg L^{-1}. The concentrations of both the original compound and its intermediate products were analyzed in quadruplicate using extraction option 4, and were found to be 9.4 μg g^{-1} for $2ØC_{10}$ LAS, and 18.3 μg g^{-1} for SPC homologues (from $5ØC_6$ to $9ØC_{10}$), with low standard deviations pf 2.2 and 3.4, respectively, thus showing the efficiency of the method developed.

Conclusions

MeOH has been demonstrated to be the most efficient solvent in the extraction of LAS (C_{10} to C_{13}) and SPCs (C_6 to C_{13}) from fish. This has enabled a new method for the simultaneous extraction, isolation, and identification of these compounds to be developed. The analytical protocol proposed utilizes PLE followed by SPE with C_{18} mini-columns, and finally identification and quantitation by LC-MS. The selectivity and reproducibility of this method is

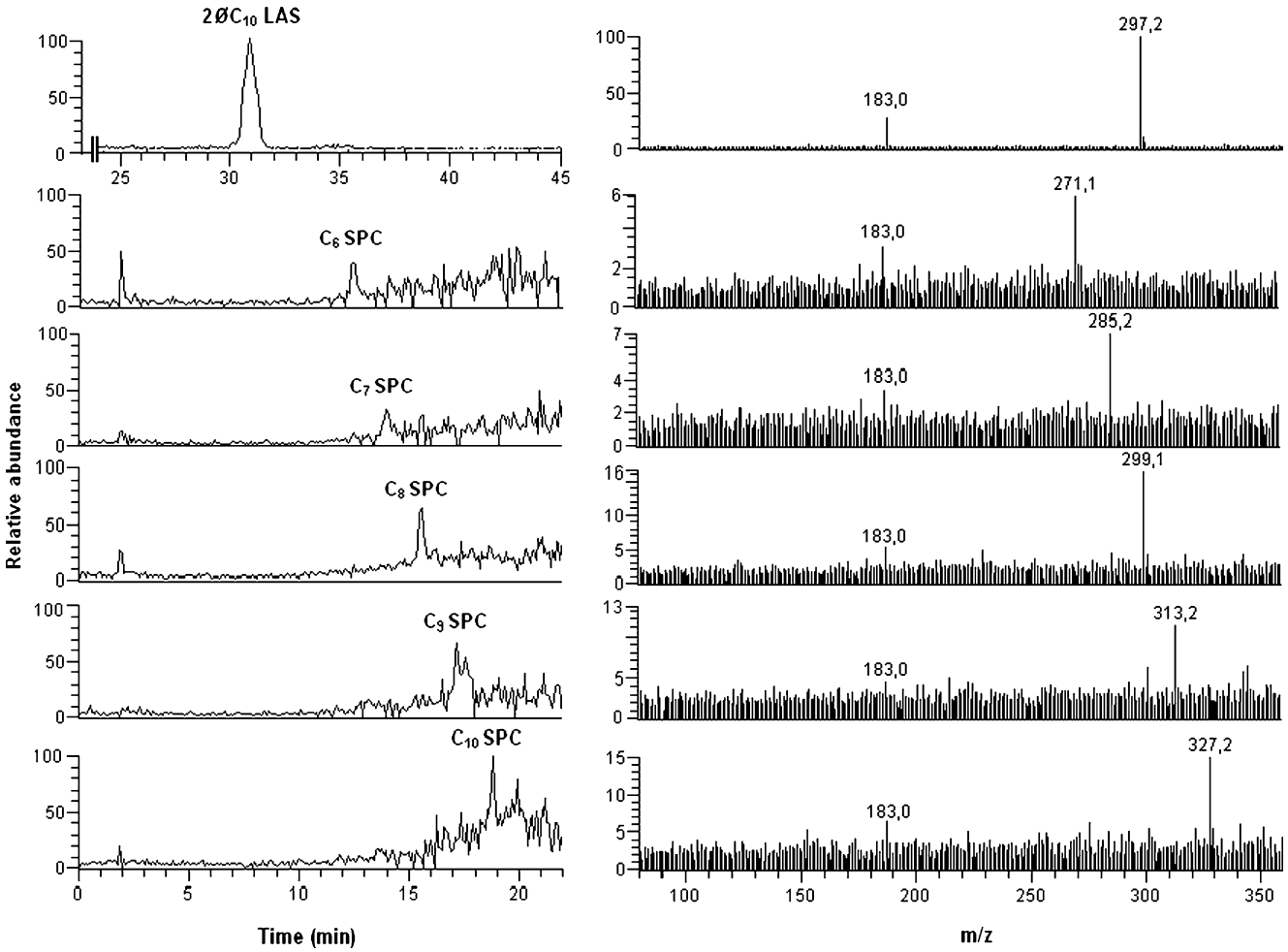

Fig. 4 LC-ESI-MS negative ion mode chromatograms and mass spectra from a *Solea senegalensis* sample arriving from a flow-through exposure system. The identification and separation of LAS and SPC homologues is showed by scanning their quasimolecular ions $[M\text{-}H]^-$

high and it is achieved with less time and less solvent consumed when compared with older methods. Moreover, the use of the LC-MS technique allows an unequivocal separation and identification of the target compounds in samples of organisms exposed in a flow-through experiment. This method has been applied to the analysis of samples of fish exposed to $2ØC_{10}$ LAS, and the concentrations of LAS and SPC homologues were determined with small standard deviations.

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