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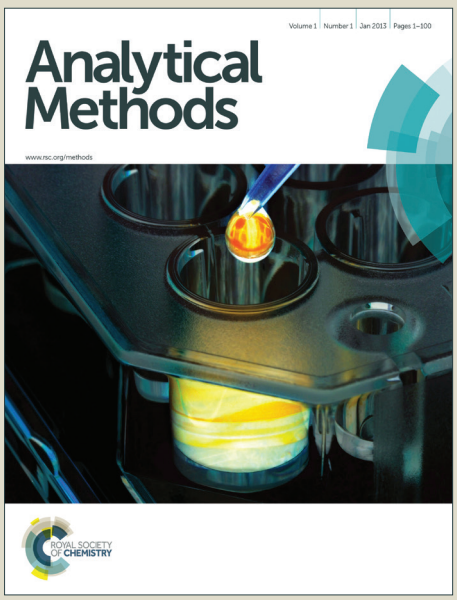

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Use of Doehlert design in the optimization of extraction conditions in the determination of organochlorine pesticides in bovine milk samples by HS-SPME

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Abstract

In this study, the extraction of the pesticides lindane, heptachlor, aldrin, dieldrin and endrin from milk samples by HS-SPME was investigated followed by separation/detection of these compounds by gas chromatography coupled to electron capture detector (GC-ECD). Due to the high complexity of milk samples and the hydrophobicity of the studied organochlorine pesticides, the samples were diluted with saturated NaCl solution prior to the optimization of the extraction conditions. Dilution tests indicated that a total sample volume of 2 mL (0.5 mL of milk and 1.5 mL of NaCl solution) provided the best chromatographic responses. The fiber coating DVB/Car/PDMS presented the best extraction efficiency towards the target compounds. Optimization was performed using a Doehlert design, and the best conditions were found to be an extraction temperature of 80°C and extraction time of 90 min. The HS-SPME mode was used for all extractions from the milk samples. A single fiber, selected during the optimization of the fiber coating, was used throughout the entire study, and no damage on coating was observed. The analytical figures of merit were evaluated, and good results were obtained for the extraction of the target organochlorine pesticides. The percentages of recovery were higher than 75% and the limit of quantitation ranged from 0.5 to 1.2 μg L^{-1} (for heptachlor and dieldrin, respectively).

Introduction

Organochlorine pesticides were widely used as insecticides in the 1950s and 1960s. Due to their toxicity, combined with their high chemical and biological stability and high degree of lipophilicity [1], this class of compounds offer advantages as pesticides. However, once entering the environment they tend to bioaccumulate in lipid-rich tissues [2]. Although the use of this kind of compound has been restricted or even banned in many countries, they continue to be found in fat matrices because of their lipophilicity [3]. Humans and other animals are mainly exposed to these chemicals through ingestion, since diet is the most important source of contamination [4], and the half-life of most organochlorine pesticides can range from a few years to more than a decade [5].

One of the most important matrices in which some organochlorine pesticides can be found is bovine milk. Bovine milk is considered a nearly complete food since it is a good source of protein, fat and major minerals. Additionally, it is a main constituent of the daily diet of many vulnerable groups, such as infants, school-aged children and the elderly [6]. Milk is an ideal liquid for dissolving non-polar environmental contaminants because of their lipophilic characteristics. Accordingly, milk can contain high levels of pesticide residues as a result of their presence in the animal tissues following cattle dipping, or due to animals feeding on possibly contaminated feedstuffs, or even drinking water which could be contaminated by pesticide residues [7].

In as much as the high toxicity of organochlorine pesticides, state bodies and environmental groups have advocated towards the international recognition in establishing strict maximum residue limits (MRLs) for pesticides in bovine milk. In the case of the European Union (EU), since September 1st 2008, a new legislative framework (Regulation (EC) No. 396/2005) for pesticide residues has been applied

[6,8]. This regulation completes the harmonization and simplification of pesticide MRLs, while aiming to protect the consumers throughout the EU [6]. The U. S. Codex online database for pesticide residues in food contains the Codex MRLs for pesticides and the extraneous maximum residue limits (EMRLs) adopted by the Codex Alimentarius Commission up to and including its 34th Session (July 2011) [6,9]. In Brazil, the MRLs for organochlorine pesticides are regulated by MAPA [10] (Ministerio da Agricultura Pecuaria e Abastecimento) and are based on the recommendations of both the Codex Alimentarius and the European Union. For instance, the MRLs established for some organochlorine pesticides are: aldrin and dieldrin 6 μg kg^{-1} (Codex, EU and MAPA); lindane 10 μg kg^{-1} (Codex and MAPA) and 1 μg kg^{-1} (EU); endrin 1 μg kg^{-1} (EU) and 2 μg kg^{-1} (MAPA) with no limit established by Codex; heptachlor 6 μg kg^{-1} (Codex) and 4 μg kg^{-1} (EU and MAPA).

In general, a fat-based matrix cannot be analyzed without some preliminary sample preparation. Milk is a complex biological matrix due to its high fat and protein contents [11]; additionally, the contaminants present are often highly diluted. These characteristics usually mean that special procedures are required for the sample preparation prior to analysis [12]. For this reason, sample extraction can be time consuming and tedious, involving several clean-up steps to remove the co-extracted material from the matrix [13]. To overcome the problems associated with these techniques (i.e., high costs, time-consuming procedures and the use of large volumes of toxic organic solvents), solid-phase microextraction (SPME) has emerged as an attractive approach. SPME has been successfully applied in the determination of organochlorine pesticides in milk samples [3], presenting great advantages when compared to the traditional techniques using solvents [14,15]. This microextraction technique was developed by Pawliszyn and coworkers [16]. TheSPME procedure is

simple, relatively quick and does not require the use of organic extractor solvents [17]. It involves the adsorption/absorption of the analytes by an adequate stationary phase coated onto a fused-silica fiber, and their subsequent thermal desorption into the gas chromatographic system for analysis [18]. Various types of polymeric coatings for extraction are commercially available, such as PDMS, PDMS/DVB, and DVB/Car/PDMS. Few studies have been reported using SPME as sample preparation step for the determination of organochlorine pesticides in milk samples [3,19,20]. Sample pretreatment with organic acids and other chemical reagents before the SPME procedure [20] as well as direct immersion (DI) mode [19] have been applied to this mean. The disadvantage of the DI-SPME procedure is the reduction of the fiber lifetime, mainly in complex samples such as milk, due to presence of fats and proteins that could be adsorbed onto the SPME extraction phase [20].

The aim of this study was to determine organochlorine pesticides (lindane, heptachlor, aldrin, dieldrin and endrin) in bovine milk samples by SPME using the headspace extraction mode, thus, allowing for extended fiber lifetime in comparison with DI mode. Furthermore, a simple dilution process before the microextraction procedures was used to improve the chromatographic responses. A Doehlert design was used for the optimization of the best extraction conditions

Experimental Procedure

Instruments

Chromatographic analyses were carried out on a Shimadzu GC-14B gas chromatograph equipped with an ^{63}Ni electron capture detector. A Restek Rtx® 5MS (30 m × 0.25 mm × 0.25 μm) separation column obtained from Restek (Bellefonte, USA) was used in this study. The oven temperature was programmed as follows: 100°C

(1 min hold), 12°C min^{-1} up to 180°C, 5°C min^{-1} up to 220°C and 3°C min^{-1} up to 250°C. Injector and detector temperatures were both set at 260°C. Ultrapure nitrogen was used as the carrier gas at 1.0 mL min^{-1}.

Reagents and materials

Extractions were performed in 15-mL headspace vials. A magnetic stirrer was used for agitation and a thermostatic bath (Nova Técnica, São Paulo, Brazil) for sample temperature stabilization. During fiber coating selection the following commercial fibers have been assessed: DVB/Car/PDMS (50/30 μm), PDMS/DVB (65 μm), PA (85 μm) and PDMS (100 μm). All fibers were obtained from Supelco (Bellefonte, PA, USA). Sodium chloride P.A. (Vetec, Rio de Janeiro, Brazil), standard solutions of the pesticides lindane, aldrin, heptachlor (20 mg L^{-1}), dieldrin and endrin (50 mg L^{-1}) in methanol (Sigma-Aldrich, Milwaukee, WI, USA) and ultra-purified water (Mega Purity, Billerica, USA) were used in this work.

Samples

Milk samples utilized in this study were obtained from supermarkets located in the city of Florianopolis, Santa Catarina State, Brazil. The sample spiking procedure was performed using a standard mixture of organochlorine pesticides. Dilution of milk samples with saturated NaCl solution was necessary to improve the chromatographic responses. After spiking with known concentrations, the milk samples were kept in a refrigerator for 24 hours at 4°C before the actual analysis.

Experimental Procedure

I) Choice of the SPME fiber

Firstly, the extraction phase was selected from the following commercially-available fiber coatings (thickness in parenthesis): DVB/Car/PDMS (50/30 μm),

PDMS/DVB (65 μm), PA (85 μm) and PDMS (100 μm). For this propose, extractions of milk samples spiked with 100 μg L^{-1} of the organochlorine pesticides were carried out using each fiber. The extractions were performed in triplicate at a sample temperature of 70°C employing an extraction time of 40 min and using a total sample volume of 2 mL under magnetic stirrer. The headspace SPME extraction mode was used in all cases to avoid damaging the fiber due to the high complexity of the milk samples. After the extraction procedure each fiber was placed in the chromatographic injector port for thermal desorption for 15 min at 260 °C. This condition ensures complete desorption of the target analytes from the fiber which contains carboxen and requires longer desorption time to avoid carryover effect.

The chromatographic peak areas were considered in order to select the most appropriate polymeric coating for this study and the analysis were performed in triplicate.

II) Dilution test

Due to the great complexity of the milk samples, it was necessary to perform some dilution tests to verify the improvement of the chromatographic responses, hence, improved sensitivity. For this purpose a saturated NaCl solution was used. Predetermined volumes of milk and NaCl solution were placed into the SPME vials. The samples were spiked with 50 μg L^{-1} of the organochlorine pesticide mixture and the vials were sealed with a PTFE/silicone septum. The total sample volume used was 2 mL, the experiments were carried out using (a) 1.5 mL of milk and 0.5 mL of NaCl satured aqueous solution, (b) 1.0 mL of milk and 1.0 mL of NaCl satured aqueous solution and (c) 0.5 mL of milk and 1.5 mL of NaCl satured aqueous solution. The samples were kept in a refrigerator at 4°C for 24 h before analysis. The extractions were

then performed using the DVB/Car/PDMS fiber, with a sample temperature of 60°C and extraction time of 40 min in the HS-SPME mode. These experiments were also performed in triplicate. The results obtained in the chromatographic analysis were compared and the best conditions determined.

III) Doehlert design to determine the optimal extraction conditions

The Doehlert matrix was used to determine the optimum extraction conditions, where extraction time (10, 37, 65, 92 and 120 min) and temperature (40, 60 and 80°C) were investigated. The factor extraction time was studied at five levels due to its wider interval than the extraction temperature, which was limited at 80°C to prevent degradation of some components of the milk. The sum of the chromatographic peak areas of the compounds was considered in order to build a response surface which allowed for the selection of the optimum experimental conditions for this study.

IV) Figures of merit and analysis of real samples

The figures of merit for the analytical method were determined. Calibration curves for target compounds were obtained; additionally, linearity, limit of detection (LOD), limit of quantification (LOQ), correlation coefficients (r), intra- and inter-day precision, as well as method recovery (%) were also evaluated. LOD and LOQ were calculated using some parameters of the analytical curve such as the estimative of standard deviation of response (s) and the slope of the calibration curve (S) [26], according to equations 1 and 2.

$$LOD = 3.3\,(s/S) \qquad \text{Eq. 1}$$

$$LOQ = 10\,(s/S) \qquad \text{Eq. 2}$$

The analytical curve was performed by spiking blank milk samples with known amounts of each analyte. In accordance with the manufacturer, milk should be left in refrigerator at 1-10°C and consumed until 48 hours. Therefore, to avoid problems with deterioration of the matrix and, at the same time, to allow interaction between the analytes and the matrix, the samples were placed in refrigerator at 4°C for 24 hours before the analysis. The recovery percentages of the analytes were determined using previously-spiked milk samples containing known concentrations of the compounds of interest at two levels, for the lower recovery levels the following analyte concentrations were employed: lindane and heptachlor (2 μg L^{-1}), aldrin (4 μg L^{-1}), dieldrin (3 μg L^{-1}), endrin (5 μg L^{-1}). For the higher recovery levels compounds concentrations were: lindane, heptachlor and aldrin (40 μg L^{-1}), dieldrin (20 μg L^{-1}), endrin (100 μg L^{-1}). These different concentration levels were used because of the different linearity ranges presented by target compounds. To evaluate precision by means of repeatability and intermediate precision, spiked milk samples containing lindane, heptachlor, endrin (1 μg L^{-1}), aldrin (4 μg L^{-1}) and dieldrin (3 μg L^{-1}) were employed. These concentrations were selected because of their proximity with the beginning of the linear range of each analyte, which represent an important region to evaluate this parameter. All analyses were performed in triplicate. In the last step, some commercial UHT milk samples obtained from supermarkets in Florianopolis were analyzed. The total fat content of the milk samples ranged between 3.0 – 4.0%.

Results and discussion

Selection of the SPME fiber coating

Several commercially-available polymeric coatings were evaluated and the chromatographic peak area obtained for the target compounds with each fiber coating

were compared. The experiments were performed in triplicate for each fiber. The results are shown in Fig. 1, where different fiber coatings used and the normalized peak areas for the target analytes are compared.

According to Fig. 1, the fiber coating composed of DVB/Car/PDMS exhibited the best extraction efficiency amongst all compared fiber coatings, except for lindane and endrin. Nonetheless, the extraction efficiency for this fiber coating towards lindane and endrin can still be considered good since it reached around 80% compared to the normalized peak areas obtained for PDMS/DVB and PA coatings (which showed the best responses for these compounds, respectively). The DVB/Car/PDMS fiber has intermediate polarity and a high extraction capacity for a wide range of compounds of different polarities [21]. Therefore, based on its extraction efficiency towards the target analytes in the present study, DVB/Car/PDMS was chosen for subsequent experiments.

Dilution tests

Considering the complexity of milk samples and the high lipophilicity of organochlorine pesticides, it was necessary to assess strategies to improve method sensitivity. One approach to minimize matrix interferences is sample dilution [22]. Therefore, a dilution study combined with modification of the ionic strength of the milk samples was performed, in order to overcome the matrix effects.

A saturated NaCl solution was used to modify the ionic strength of the milk samples while concomitantly performing the dilution.

In this regard, dilution tests were carried out using a total sample volume of 2 mL, while the volume of milk and NaCl solution were varied, as described in the experimental procedure section, and microextractions were performed for 40 min at

70°C. Figure 2 shows a bar graph with the normalized peak areas for each compound studied in three different degrees of dilution, the analysis were performed in triplicate.

As can be seen in Fig. 2, the dilution of the milk samples with saturated NaCl solution had a significant effect on the response, thus, extraction efficiency. An increase in dilution ratio leads to improved chromatographic response for all target compounds. The best results were found using 0.5 mL of milk and 1.5 mL of NaCl solution.

The addition of salt increased the ionic strength of the solution, changing the vapor pressure, viscosity, solubility, density and surface tension of the solution containing the analytes, which in turn results in a change in the liquid/vapor equilibrium of the system [23]. In this case, the increase in the chromatographic response can be explained by the modification of one or more of the abovementioned properties induced not only by the addition of salt to the milk samples but also affected by sample dilution.

As a consequence of this optimization, the matrix effect was significantly decreased and higher values for the chromatographic peak areas were obtained for all target compounds.

The dilution of the samples has been shown to be effective at reducing matrix interference, creating cleaner chromatographic background, therefore, facilitating the detection of analytes present at low concentrations.

The chromatogram shown in the Fig. 3 was obtained using the optimized conditions previously determined in the dilution study, 0.5 mL of milk sample spiked with the organochlorine pesticide mixture and diluted with 1.5 mL of saturared NaCl solution, using the DVB/Car/PDMS fiber, an extraction time of 40 min and an extraction temperature of 70°C, and performing the extraction in the HS mode.

Optimization of extraction time and sample temperature by Doehlert design

One of the most important steps in developing the SPME methodology is the determination of the best experimental conditions. For this optimization the extraction temperature and extraction time were evaluated. In relation to the extraction temperature, an increase in this value aids the transfer of analytes from the condensed phase to the headspace and also increases the volatility of non-volatile analytes by increasing their diffusion coefficients. At elevated temperatures, native analytes can effectively dissociate from the matrix and move into the headspace for rapid extraction by the fiber coating. However, the coating/sample distribution coefficient may also decrease with an increase in temperature, resulting in a diminution of the analyte extracted underequilibrium [24]. This occurs because the sorption process is exothermic in nature. For gaseous samples, the temperature must be high enough to prevent analytes from condensing, but not so high that the partition coefficient decreases [25]. As the SPME is a measure of the free concentration of analytes in samples, rather than total concentration, it is characterized as an equilibrium extraction technique. Another important factor to be evaluated in the SPME procedure is the extraction time, which always involves a compromise between the sensitivity and repeatability of the analytical method. A suitable extraction time allows not only a sufficient mass of analyte to reach the surface of the extraction phase but it also includes the time required for the analyte to reach the boundary layer and for its diffusion within the boundary layer surrounding the extraction phase. Depending on the physicochemical properties of a given analyte, when the extraction occurs from the headspace above a liquid or solid sample, the mass transfer from the liquid or solid to the headspace may be slower compared to extractions performed in the direct immersion mode [24].

Given the abovementioned interaction between extraction time and sample temperature, in the present study the optimization of the extraction temperature (40 - 80 °C) and time (10 - 120 min) for the HS-SPME procedure was performed using the Doehlert matrix.The sum of the chromatographic peak areas for all target compounds obtained in each experiment was used to build a response surface to verify the best conditions in this optimization, which is shown in Fig. 4.

It can be observed through the response surface methodology that good results for the organochlorine pesticide extraction were obtained with a temperature of around 80°C and extraction time of approximately 90 min. Also can be observed from Fig. 4 that extraction efficiency is still increasing with higher extraction time and sample temperature. However, regarding with the possibility of the water evaporation at higher temperatures and consequent problems associated to vapor pressure inside the SPME vials, the value of 80 °C was selected. In relation of the extraction time, it was observed that 90 min already showed good chromatographic responses, avoiding an unnecessary long extraction time. These relatively high values for both variables, in the SPME procedure, may be easily interpreted according to these compound`s physicochemical properties. As the milk samples studied contained 3.0-4.0% of total fat and the fact that organochlorine pesticides are lipophilic compounds, higher values for the extraction time and extraction temperature are required to improve the HS-SPME sensitivity. Indeed, such target compounds are characterized as semi-volatile compounds, and in addition to their high affinity for some components present in the matrix one could expect that a higher temperature would be necessary in order to successfully drive these analytes to the headspace above the sample.

Determination of analytical parameters of merit and analysis of real samples

After the optimization steps, the optimal conditions were used to determine the analytical parameters of merit. The analytical curves were obtained from spiked milk samples with known concentrations of the target compounds. Each analytical curve was obtained using at least 5 concentration levels of the organochlorine pesticides. Several important figures of merit were evaluated and some results obtained are shown in Table 1.

In Table 1, it can be observed that good correlation coefficients (r) were obtained for the organochlorine pesticides (0.9755 – 0.9997), indicating that good linearity was obtained with the proposed method, the linear ranges varied from 1-100 μg L^{-1} to endrin, 2-40 μg L^{-1} to lindane, heptachlor and aldrin; and 3-20 μg L^{-1} to dieldrin. . Regarding to the limits of detection and quantification for the method, these values were obtained using the parameters of the analytical curve as described in the experimental section. The LOQ values ranged from 0.5 μg L^{-1} for heptachlor to 1.2 μg L^{-1} for dieldrin. The obtained LOQs are not only satisfactory and comparable with other values reported in the literature for milk samples, but also compliant with the established MRLs [3]. Other important parameters evaluated were the repeatability and the intermediate precision. The intraday precision or repeatability represents the agreement between successive measurements obtained using the same method and the same conditions (regarding to the analyst, location and equipment and measured within a short period of time) [26]. The values obtained for this parameter ranged from 2.1% for endrin to 22.2% for heptachlor and they can be classified as satisfactory given the complexity of the sample matrix and the concentration levels assessed. The intermediate precision or interday precision indicates the effects of variations within the laboratory due to events like different test days, analysts or equipment or a mixture of these factors [26]. Thus, in this study measurements were also performed on different days and good

results for the intermediate precision were obtained, ranging from 7.2% for lindane to 24.6% for heptachlor.

The percentage recovery of these pesticides using different concentrations was also evaluated. Within the linear range, concentrations bracketing the lower and the upper limits of linearity were chosen, corresponding to the lower and higher levels of recovery studied.

It can be observed that the recovery percentages obtained were satisfactory (ranged from 76% for aldrin to 120% for heptachlor) for both levels, which is an important factor in trace analysis, especially when working with complex samples, such as milk.

Finally, the analysis of commercial samples obtained from supermarkets in the city of Florianopolis was carried out. The same conditions used to build the analytical curves were applied in this step: extraction time of 90 min, extraction temperature of 80°C, and total sample volume of 2 mL (1.5 mL of saturared NaCl solution and 0.5 mL of commercial UHT milk samples). Three different commercially-available full-fat milk samples were analyzed, with total fat contents ranging from 3.0 to 4.0%. In the analysis of the real samples the organochlorine pesticides studied were not detected, verifying the good quality of the commercial samples regarding the presence of these types of contaminants.

Conclusions

The use of the HS-SPME mode for the determination of organochlorine pesticides in milk samples was successfully implemented. The present method issensitive, efficient and does not require the use of organic solvents. Sample dilution, modification of ionic strength , and the Doehlert optimization were found to be very paramount in the acquisition of robust and sensitive method. Moreover, HS-SPME is

particularly useful when dealing with complex samples given the possibility of the SPME fiber being damaged in the direct immersion mode. Differently from DI-SPME that can have fiber lifetime shortened due to fouling of fiber surface by matrix macromolecules [3], in HS-SPME the fiber coating is protected from damaged, which allowed the use of a single DVB/Car/PDMS fiber throughout the whole of the present study, and no evidence of its damage was observed.

Regardless the complexity of milk samples and lipophilicity of organochlorine pesticides, good limits of detection and quantification were obtained for the 5 pesticides studied according to the current MRLs. Moreover, good correlation coefficients were obtained bracketing the MRLs, demonstrating not only the good linearity of the method but also its applicability to ensure that commercial samples are compliant with current legislation.

Acknowledgements

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Tables and figures captions

Table 1: Figures of merit for the organochlorine pesticides.

Figure 1: Different commercial coatings used in the fiber optimization, the extractions were performed in triplicate, the target compounds as follows: 1) lindane, 2) heptachlor, 3) aldrin, 4) dieldrin, 5) endrin.

Figure 2: Bar graph showing the normalized peak areas for the target compounds: 1) lindane, 2) heptachlor, 3) aldrin, 4) dieldrin and 5) endrin. Using a total sample volume of 2 mL, the volume removed was replaced with the saturated aqueous solution of NaCl. The samples were analyzed in triplicate and the relative standard deviation was below 20% for all compounds.

Figure 3: Chromatogram obtained by GC-ECD for spiked milk sample. The elution order was as follows: 1) lindane (40 μg L^{-1}); 2) heptachlor (40 μg L^{-1}); 3) aldrin (40 μg L^{-1}); 4) dieldrin (100 μg L^{-1}) and 5) endrin (100 μg L^{-1}).

Figure 4: Response surface obtained from the Doehlert design in the optimization of the extraction time and extraction temperature.

Table 1.

Compound	Linear range ($\mu g\ L^{-1}$)	Correlation coefficient (r)	LOD ($\mu g\ L^{-1}$)	LOQ ($\mu g\ L^{-1}$)	RSD[a] (%)	RSD[b] (%)	Recovery Lower level (%)	Recovery Higher level (%)
Lindane	2.0-40.0	0.9936	0.2	0.6	10.1	7.2	115	103
Heptachlor	2.0-40.0	0.9946	0.2	0.5	22.2	24.6	120	102
Aldrin	4.0-40.0	0.9903	0.2	0.7	5.8	8.3	76	96
Dieldrin	3.0-20.0	0.9755	0.4	1.2	13.2	22.1	110	98
Endrin	1.0-100.0	0.9997	0.3	1.0	2.1	19.4	109	99

RSD[a] – intraday precision (repeatability) of spiked milk samples containing lindane, heptachlor, endrin (1 $\mu g\ L^{-1}$), aldrin (4 $\mu g\ L^{-1}$) and dieldrin (3 $\mu g\ L^{-1}$). (n=3)

RSD[b] – interday precision (intermediate precision) of spiked milk samples containing lindane, heptachlor, endrin (1 $\mu g\ L^{-1}$), aldrin (4 $\mu g\ L^{-1}$) and dieldrin (3 $\mu g\ L^{-1}$). (n=3)

Recovery - Lower level: lindane and heptachlor (2 $\mu g\ L^{-1}$), aldrin (4 $\mu g\ L^{-1}$), dieldrin (3 $\mu g\ L^{-1}$), endrin (5 $\mu g\ L^{-1}$).

Recovery - Higher level: lindane, heptachlor and aldrin (40 $\mu g\ L^{-1}$), dieldrin (20 $\mu g\ L^{-1}$), endrin (100 $\mu g\ L^{-1}$).

Figure 1

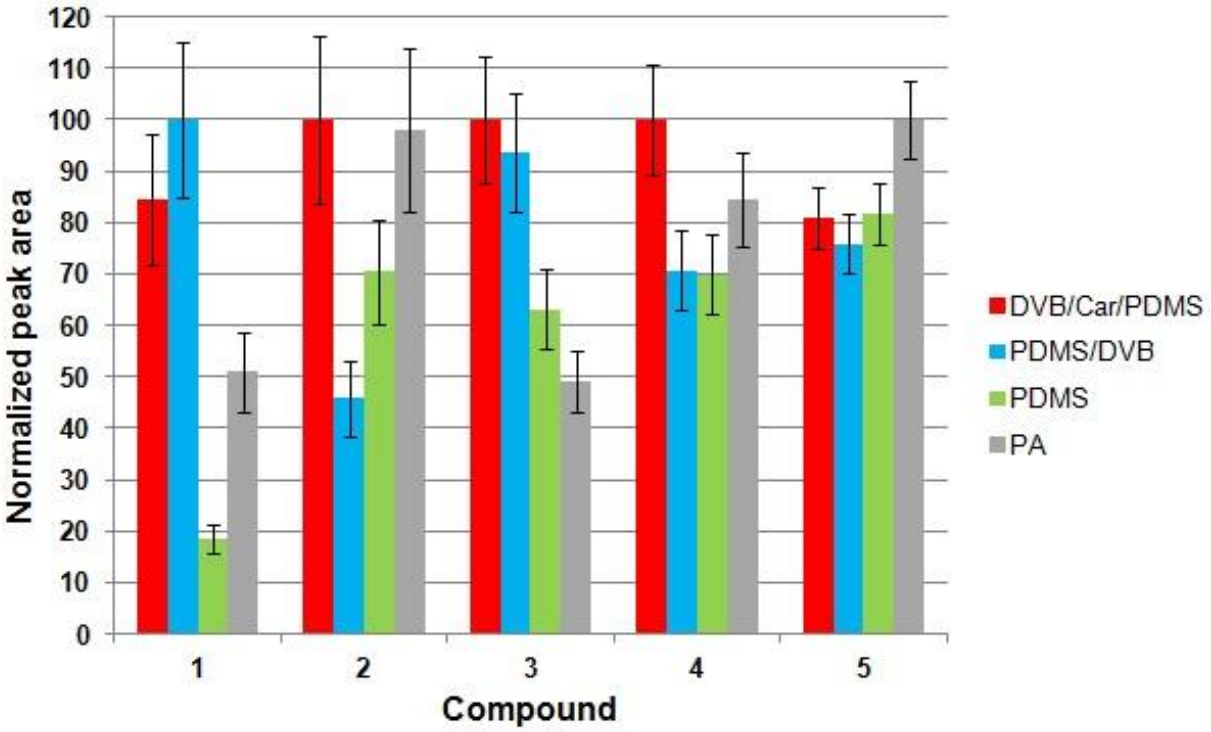

Figure 2

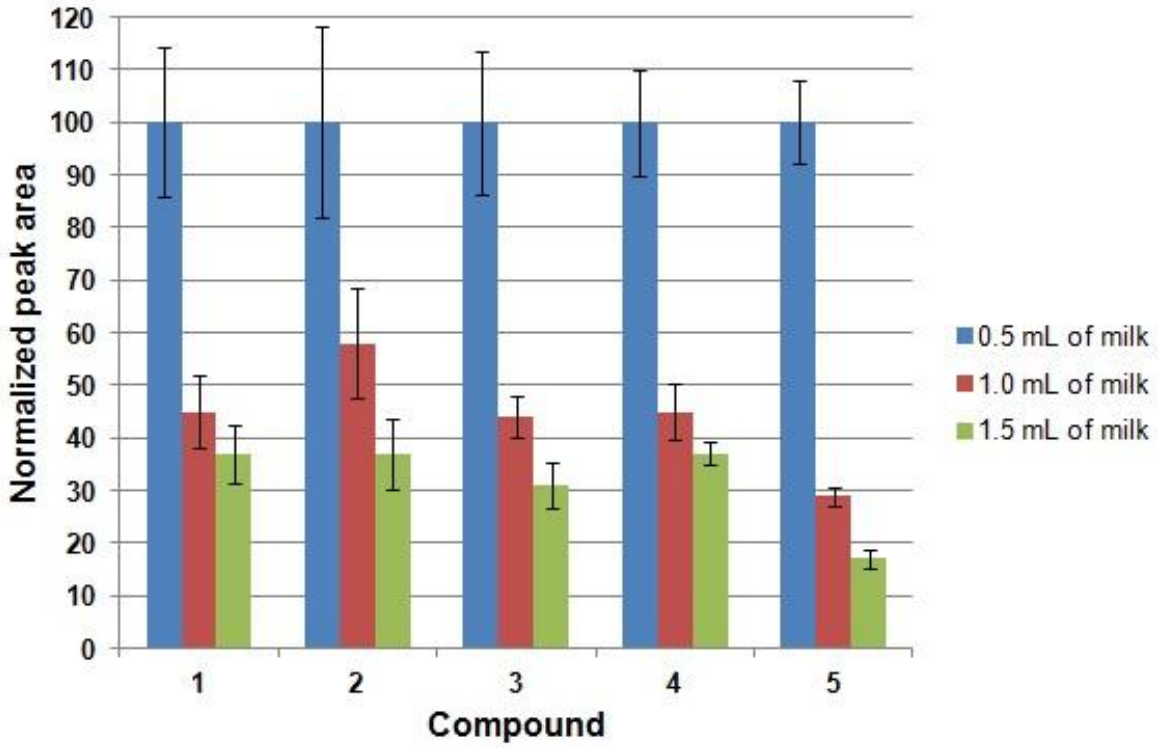

Figure 3

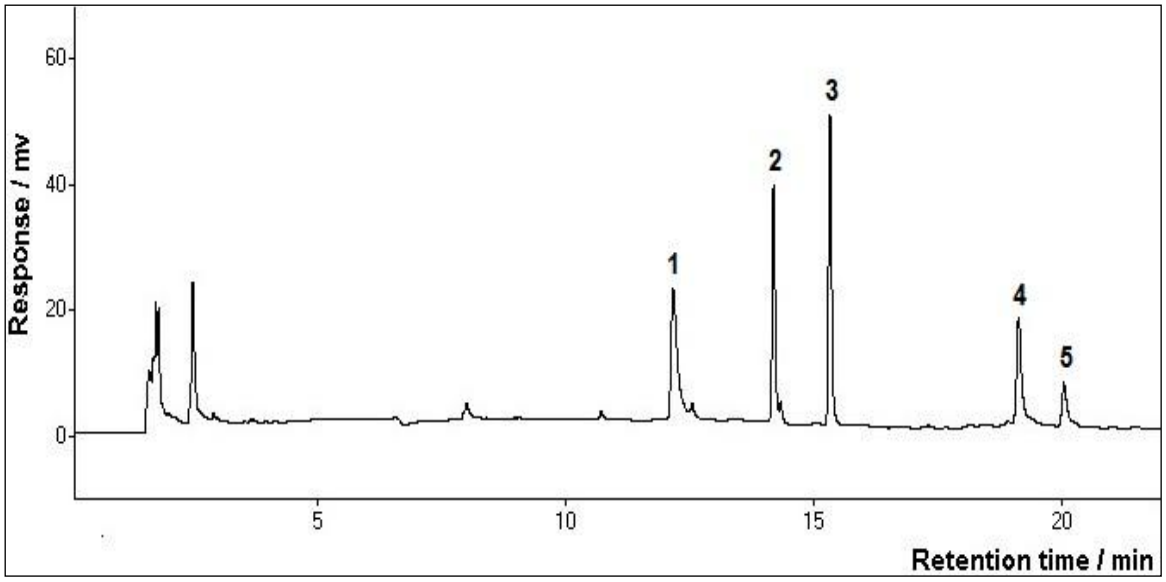

Figure 4