New home-made assembly for hollow-fibre membrane extraction of persistent organic pollutants from real world samples

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ABSTRACT. Nowadays, hollow fibre membrane extraction techniques are widely used but they are usually applied to water or very simple matrices such as water. In this paper, we propose a new assembly that allows the extraction of forty persistent organic pollutants in real world samples, namely orange juice, porcine plasma and tomatoes. The limits of detection obtained are very low even in the analysis of real samples (9-182 ng L^{-1}). The relative standard deviations varies from 1-18 % and the averaged recoveries in the spike experiments are very high (65-120 %) in the different types of samples studied. The new assembly allows a very good precision overcoming one of the most important shortcomings of membrane extraction techniques. A central composite design has been performed to get optima extraction conditions for the analytes and also the combined response of all the analytes has been obtained to attain the simultaneous optimum.

KEYWORDS. Membrane extraction, hollow-fibre, gas chromatography, electron capture detector, mass spectrometry, fruit juice, tomato, plasma.

1. Introduction

The first application of flat sheet membranes in analytical chemistry was developed in 1963 and about ten years later, the use of a hollow fibre membrane was described, in both cases the membranes were used for membrane introduction mass spectrometry (MIMS) [1]. Nowadays, membrane based extraction techniques have become a promising alternative against canonical analytical extraction techniques or more recent miniaturised techniques (solid phase microextraction (SPME) and liquid phase microextraction (LPME)) in different modes: single-drop microextraction (SDME), dispersive liquid-liquid microextraction (DLLME) and hollow-fibre microextraction (HF-LPME) [2]. The major problem of LPME is that the microdrop suspended on the microsyringe needle is easily dislodged during stirring and moreover it is not suitable for dirty samples because particles make the drop unstable [3]. The HF-LPME introduced by Pedersen-Bjergaard and Rasmussen overcomes problems related with the application to dirty samples such as sample carryover and reproducibility [4].

The HF membrane extraction techniques are mainly related with the use of hydrophobic membranes that are porous or non-porous polymeric materials. A great number of publications related with the use of porous membranes describes different modes: (i) hollow fibre and flat sheet microporous membrane liquid-liquid extraction, (ii) two phase hollow-fibre liquid phase microextraction, (iii) membrane protected solid-phase microextraction and solid phase extraction, (iv) polymer-coated hollow fibre membrane microextraction [1] and (v) hollow fibre liquid-liquid-liquid microextraction [3]. The extraction principles and historical developments of LPME with porous hollow fibres have been discussed [5]; as well as the applications in environmental and bioanalytical issues [6]. Miniaturised membrane based extraction techniques have several important advantages such as reproducibility, absence of sample carryover (due to the disposable nature of the membranes), high analyte enrichments,

high throughput, low cost, applicability to many different types of analytes, large pH tolerance range and facility for automation and conversion into greener analytical techniques.

The concern about the contamination by persistent organic pollutants (POPs) has risen over recent decades. The action of pesticides as hormone endocrine disruptors and cancer inducers is well known [7]. The persistent character and propensity to accumulate in biological tissues of pesticides and polychlorinated biphenyls (PCBs) have also been established [7,8], these latter used as flame retardants. Trace amounts of PCB congeners have also been associated with higher incidence of foetal miscarriage [9]. The physicochemical properties of polybrominated diphenyl ethers (PBDEs) are similar to PCBs, they are toxic, persistent and lipophilic [10]. Therefore, POPs are ubiquitous pollutants and currently their analysis in environmental and food samples is growing due to the marked increase in the levels of these compounds during the last decade [11,12]. Several works have been published in relation with the use of membrane extraction techniques for POPs. In this sense, HF-LPME has been used for the determination of organochlorine pesticides in seawater [13] and water [14,16] for the extraction of PCBs in blood plasma [9] and PBDEs in environmental waters [17].

In the present paper we have developed a new home-made assembly for the extraction of about 40 POPs including pesticides, PCBs and PBDEs using the hollow-fibre membrane extraction technique. The approach is simpler than those proposed in the literature and the analytical features, especially reproducibility and sample throughput improve previous proposals. The approach has been optimised using a rotable central composite design (RCCD) and the validation has been carried out on real world samples as fruit juice, tomatoes pulp and porcine plasma.

2. Materials and methods

2.1. Standard solutions, reagents and samples

All reagents used were of highest purity. Isooctane, cyclohexane, n-decane, acetonitrile, methanol and toluene were obtained from Teknokroma (Barcelona, Spain). Ultrapure water (18 MΩ cm) was obtained from a Milli-Q water-purification system and was used throughout (Millipore, Watford, UK).

The pesticides (β-BHC, γ-BHC, δ-BHC, α-endosulfan, β-endosulfan, heptachlor, heptachlor epoxide, aldrin, endrin, dieldrin, chlorthalonil, α-cypermethrin, biphentrin, acrinathrin, fenarimol, phosalone, 4,4'-DDD, 4,4'-DDT, 4,4'-DDE, λ-cyhalothrin) were obtained from Sigma–Aldrich (Steinheim, Germany). The PCBs (PCB#1, 5, 29, 28, 52, 47, 98, 154, 153, 138, 171, 200) were supplied by Sigma–Aldrich (Steinheim, Germany). The PBDEs (PBDE#28, 47, 85, 99, 100, 153, 154, 183) were obtained from Fluka and Accurel (Sigma–Aldrich (Steinheim, Germany).

Stock standard solutions of 1000 mg L^{-1} were prepared dissolving the standards in acetone. Working solutions for direct injection were prepared daily by stepwise dilution of their stock solutions with n-Hexane. Spiking solutions were prepared in acetone. All the standard solutions were stored at 4ºC in darkness until the analysis.

Samples of orange juice, tomatoes and porcine plasma samples were purchased from a local market.

2.2. Extraction of POPs using the home-made assembly for HF-LPME

The HF-LPME assembly designed for the extraction of POPs is shown in Figure 1. The porous hollow fibre used to support the organic phase and for containing the acceptor solution was Q3/2 polypropylene (Accurel Q3/2, Membrana, Wupertal, Germany) with an internal diameter of 600 µm, 200 µm of wall thickness and 0.2 µm pores. The assembly consists of an Eppendorf GELoader pipette tip for

filling microinjection capillaries with a volume range of 0.5-20 µL (Eppendorf, Hamburg, Germany), 2.6 cm of HF and a 4 mL vial (Supelco, Bellefonte, USA) covered with a septum (20 mm diameter and 3 mm thickness, PTFE/silicone, Supelco, Bellefonte, USA). First of all the HF is cut and one end closed by means of a hot soldering tool. The pipette tip end is cut allowing a perfect connection with the open end of the membrane and then, the HF is introduced in the extraction solvent (toluene) during 1 minute to open the pores. Using a 500 µL volume GC syringe (Hamilton, Supelco, Bellefonte, USA) the air is removed from the lumen of the membrane and filled with the solvent contained in the vial. Immediately, the HF-tip pipette assembly is introduced in a vial containing 4 mL of sample (a few millimetres below the sample surface). The extraction is carried out during 37 minutes at 46 ºC and 1100 rpm. After that, the sealed end of the HF is cut and 1 µL of toluene with the analytes is extracted from the membrane through the tip of micropipette using a 10 µL syringe for the GC analysis.

The extraction of food samples was directly performed on freshly squeezed tomatoes, commercial orange juices and porcine plasma.

2.3. Gas chromatographic analysis

The extracts were analysed on a Varian Model 3800 gas chromatograph coupled to a Saturn 2000 ion-trap mass spectrometer detector (Varian, Sunnyvale, CA, USA). The gas chromatograph was fitted with a silica capillary column with a VF-5ms stationary phase and dimensions: 30 m x 0.25mm I.D, 0.25 µm film thickness (Factor Four CPSIL-8, Varian Iberica). 1 µL of the extract was injected into a split-splitless injector, operated in the splitless mode (splitless time: 45 s). The temperature was fixed at 250 ºC into the injector oven. Carrier gas was helium at a flow-rate of 1.3 mL min^{-1}. The temperature of the GC-MS transfer line was maintained at 280 ºC. The temperature was programmed in the oven of the GC as follows: from 75 ºC (held for 1 min) subsequently increased to 150 ºC with a ramp of 30 ºC min^{-1}, to 180 ºC with a ramp of 5 ºC min^{-1} (held for 15 min) , to 190 ºC with a ramp of 5 ºC min^{-1} (held for 5 min), to 200 ºC with a ramp of 5 ºC min^{-1} (held for 3 min), to 280 ºC with a ramp of 5 ºC min^{-1} (held for 2.5 min) and finally to 290 ºC with a ramp of 50 ºC min^{-1} (held for 4.8 min). The total chromatographic run

115 time was 60 minutes. The MS-MS detection under electron impact (EI) conditions are summarised in
116 Table 1.

117 The extracts were simultaneously analysed in a 6890N GC-μECD (Hewlett Packard (HP),
118 Wilmington, USA). A Chrompack CP-SIL8 chromatographic column (30m × 0.25mm × 0.25 μm) was
119 used. The oven temperature program, injector temperature and helium flow-rate were the above
120 described. The make up gas was N_2 at 40 mL min^{-1} and detector temperature was set at 300 ºC.

121

122 **2.4. Experimental design and data statistical analysis**

123 The experimental design used in this paper is based on other described previously and published
124 elsewhere [18,19]. For the optimization of the operating variables affecting the HF-LPME, a full second-
125 order central composite design (CCD) was applied. The needed computations were performed by using
126 the STATISTICA (StatSoft, Tulsa, USA) and some home-made programs written in QUICKBASIC.

127

128 # 3. Results and discussion

129

130 **3.1. Development of a home-made assembly for HF-LPME**

131 From our point of view, the real shortcoming of HF-LPME is the requirement of very skilled
132 chemists due to extraction assembly miniaturization and the lack of commercial prototypes. As a
133 consequence, the reproducibility of the procedure can be negatively affected. Our assembly (Figure 1)
134 overcomes this problem by using a membrane supported on a GELoader pipette tip that can be cut to fit
135 perfectly with the HF (the inner diameter of membrane can be exactly fitted to the external diameter of
136 pipette tip). The simplicity and reproducibility of this assembly represents an important improvement

with respect to other ones based on the use of syringe needles. Under our experience, with this later the HF is frequently lost from the needle during handling, e.g. during stirring of sample solution in the vial or insertion/removal of HF into the vial. In addition, leaking of the fibre is frequently observed.

On the other hand, we do not observe losses during the extraction without sealing the vial. Therefore, the procedure can be substantially simplified by using a septum covering the vial to support the tip connected to the HF. Finally, the introduction of the extraction solvent into the fibre is performed by the immersion of the pipette-tip/fibre assembly into a vial containing the solvent, suctioning with a syringe. This procedure avoid the introduction of bubbles into the membrane and the incomplete filling of the pores and lumen of the fibre with the extraction solvent, which is a frequent drawback when the solvent is directly introduced into the fibre using a syringe.

3.2. Optimization of the gas chromatographic separation and MS detection

The chromatographic separation of the analytes considered is a difficult task due to the great number of compounds and especially the different chemical behaviour of pesticides against PCBs and PBDEs. Finally, a good separation was achieved under conditions described in the Experimental section. A typical chromatogram showing the separation of all the analytes with direct injection is shown in Figure 2. A remarkable advantage of the chromatographic approach is the combination of the sensitivity of electron capture detector with the selectivity of the mass spectrometer to get both low detection limits and unequivocal identification of the analytes.

3.3. Optimization of HF-LPME extraction

3.3.1. Screening factors and preliminary choices

The most important variables that affect the HF-LPME extraction are the following: type of extraction solvent, HF length, immersion and extraction time, percentage of other organic solvent in the sample, stirring speed, temperature and ionic strength. For a preliminary screening of critical variables an univariate optimization of some of them was carried out that were set as follows: temperature (25 ºC), HF length (1.5 cm), immersion time (1 min), ionic strength (0% NaCl), extraction time (5 min), percentage of other organic solvent in the sample (0%) and sample volume (4 mL).

Four different types of solvents were tested for the extraction of POPs: isooctane, cyclohexane, n-decane and toluene. The results obtained are shown in Figure 3. As can be seen, the most suitable extraction solvent is toluene for the majority of the analytes, so this solvent was selected for further experiments. An immersion time of 1 minute was considered enough to open the pores of the membranes because longer times do not increases the extraction yield.

The extraction process is based on analyte diffusion from the sample to the acceptor solvent inside the HF. For this reason, stirring of sample could reduce the time to get the partition equilibrium. Stirring speed from 500 rpm to 1400 rpm were assayed and results are shown in Figure 4. It can be observed that increasing stirring speed enhances extraction yielding of all the analytes, although air bubbles appear inside the membrane for high stirring speed that are detrimental for analysis reproducibility. Therefore, a compromise value was set at 1100 rpm for further experiments.

The addition of organic solvents to the sample can increases the extraction efficiency [20,21]. For this purpose both acetonitrile and methanol have been added at different percentages (0, 10, 20, 30

%) to the sample. The addition of acetonitrile provokes decreasing of chromatographic peaks areas and methanol does not affect significantly the analyte extraction (Figures 5 and 6). Therefore, the use of additional solvents was removed from the extraction procedure. On the other hand, temperatures higher than 50 ºC provoke important loses of the solvent contained in the lumen.

Finally, increasing of ionic strength affects the diffusion coefficients of the analytes [9,23] and for this reason the presence of NaCl in the sample was studied from 0 to 30 % (w/v), although this factor does not contributes significantly to the extraction of the analytes and was not considered in further experiments.

From the univariate optimization we also select the domain of variables later optimised using a multivariate approach as follows: temperature (30-50 ºC), extraction time (5-45 min) and membrane length (10-30 cm).

3.3.2. Multivariate optimization

For a better understanding of the influence of the variables in the extraction of POPs by HF-LPME, a rotable second-order central composite design (RCCD) was built considering three variables: extraction time (X_1), temperature of the extraction (X_2) and HF length (X_3). The CCD introduced by Box and Wilson [23] consists in a 2^k (k= number of variables) full factorial design augmented with 2k vertices of a cross-polytope (star points) positioned on the coordinate axes of the factorial space and C (number of replicates) points at the center of the design, (0,0,…0). For three factors, the resulting points are (±α,0,0), (0, ±α,0) and (0,0, ±α), where α is the distance (star arm) from the centre of the design to a star point. This implies performing 2^k+2k+C experiments. In the present work, it has been applied a rotable CCD that was introduced by Box and Hunter [24] in which the variance of the predicted response values depends only on the distance from the center of the design and $\alpha = 2^{k/4}$ (k = number of variables).

In this study, each variable has five coded levels (-α, -1, 0, 1, α) to carry out the optimization. Star arms -α and α are associated to the minimum and maximum value of the factor in the common working range. Table 2 shows the experimental matrix design of the RCCD (α = 1.68) with real and

coded levels. The central point was run thrice (C = 3) for the sake of experimental error estimation and the RCCD involved 17 experiments. The experiments were randomly performed to avoid any systematic errors. Table 3 gathers the response (peak areas) of the POPs obtained in the different experiments (RCCD) carried out by HF-LPME for the optimization.

The results for the experimental design were subjected to multiple regression to obtain the coefficients of the second order polynomial (Equation (I)), where "y" is the experimental response, β the coefficients of the equation, and Xi and Xj the coded levels of the variables i and j, respectively. A significant assay for the regression coefficients was performed based on the Student t-test once the standard deviation of the regression coefficients bk, s(bk), is known. In this test, the significance of the effect is demonstrated when its absolute value (Equation (II) is found to be greater than the critical tabulated value tcrit (υ, P) for the υ degrees of freedom corresponding to the tested regression model (υ = number of runs-number of coefficients to be estimated) at a P confidence level of 95%.

The reduced models representing the relationship between variables and responses are collected in Table 4. As we can see, all the variables have significant effects in the response of all the analytes and the effect is positive as well. The squared terms of the variables are significant in the response of some analytes, especially for pesticides, that indicated a high effect in the response. In the majority of the cases, the interaction between the extraction time and the temperature has a positive effect in the peak area of the analytes. Also the interaction between the temperature and the HF length is present in many cases as a positive effect. In few cases, the interaction of the extraction time with the HF length has a positive significant effect in the peak areas of the analytes.

We consider of great interest to develop a method optimised for all the persistent organic pollutants simultaneously. For this reason, we decided to combine the responses of all the analytes for a better optimization since the corresponding models are entered without neglecting any term to avoid intermediate rounding errors. Thus, the goal of the optimization is the simultaneous maximization of the peak areas of all the POPs. The combined response has been previously defined and published elsewhere

[25]. Briefly, the combined response is a response variable created as a weighed sum of several responses according to the (equation III), where w_i is a user chosen weight, generally set as unity, Yi is each response to be optimised and R_i is the range of Y_i. When the responses in the combined response are to be maximised, then k = 1 and G_i = minimum $\{Y_i - ½ R_i\}$. By using a home-made routine algorithm, a random search of the surface grid COMB(X_1,X_2,X_3) is performed. The maximum of the COMB surface is the simultaneous optimum.

The COMBINED 3D surface shape against the axis time and length is plotted in Figure 7, and the corresponding contour plot showing the optimum point is displayed in Figure 8. As can be seen, the optimum value is reached at the coordinates time = 1, temperature = 1 and length = 1. The optimum values of the variables resulted to be: 37 min of extraction time, 46 ºC and 26 mm of HF length.

3.4. Parameters of quality of extraction method

To probe the applicability of our proposed extraction approach to real samples the parameters of quality have been calculated in real world samples, namely: orange juice, tomato pulp and porcine plasma. Results obtained are summarized in Table 5. Linear calibration curves were obtained from quantification limits to 10 μg L^{-1} for all the analytes showing very good correlation coefficients (R> 0.997, for all the analytes). Detection limits were calculated as the concentration of analyte to yield a net peak at three times the standard deviation of the background signal (3σ). The detection and quantification limits (LODs and LOQs, respectively) were calculated with the data generated in the linearity studies. After reshaping the calibration function, LOD and LOQ were calculated as the analyte concentration that corresponds to a signal equal to “a + 3 Sy/x” and “a + 10 Sy/x” respectively, where “a” is the origin ordinate, and “Sy/x” indicates the random errors for the slops. The detection limits ranged from 15 to 106 ng L^{-1} (Table 5), which are very low in comparison with other results in literature.

To evaluate the enrichment factor obtained with the HF-LPME assembly the equation IV was used, where V_a is the volume of aqueous phase (4 mL), V_d the volume of organic phase (20 µL of toluene), and $C_{eq\ a}$ and $C_{eq\ d}$ the concentration of analyte in the aqueous and organic phases, respectively. The enrichment factors for 500 ng L^{-1} have been calculated for all the analytes and the data are presented in Table 5. We can observe that the enrichment factors are around 100 fold that is in good agreement with those published in the literature. This fact led as to conclude that the technique is really efficient for the extraction of POPs.

The relative standard deviation (% RSD) of enrichment factors were obtained for five sequential injections of the analytes at two different concentrations (0.5 µg L^{-1} and 1 µg L^{-1}) in water and the results are in the range of 8-15%.

3.5. Application to real world samples

The proposed procedure has been applied to real world samples, namely, orange juice, tomatoes pulp and porcine plasma. For this purpose, recovery experiments were carried out in samples fortified at two different concentrations 500 and 750 µg L^{-1} and Table 6 shows the results obtained. The relative standard deviations (n=3) ranging from 4-17 % shows the very good reproducibility of the proposed approach. The averaged recoveries in the spike experiments were very high in the different types of samples studied (orange juice, tomatoes and porcine plasma). As we can see, this work demonstrated the very high performance of the analytical extraction approach based on the HF- LPME assembly developed when it is applied to complex matrices, such as food and biological samples that is the real shortcoming of this extraction technique, usually applied in the literature to the analysis of more simple samples like water.

Conclusions

In this paper a new assembly for hollow-fiber membrane extraction of forty POP´s from real world samples was designed. The POP´s determined in this study covers a wide number of pesticides, PCB´s and PBDE´s and the optimization has been performed to get the best conditions for the simultaneous analysis of all of them. By applying chemometric approaches based on the use of experimental design methods, the optimal extraction conditions corresponding to the highest recovery were calculated. The statistical approaches described in this paper provide useful tools for the simultaneous optimization of all the responses which is not commonly considered in the majority of similar studies. The proposed approach provides limits of detection in the range of ng L^{-1}, recoveries around 100 % for most of the analytes and enrichment factors that are very high in comparison with other published papers that applied the procedure only to very simple aqueous matrices. The most important parameter of quality is the precision since with the proposed approach is possible to attain a %RSD from 4-17 % for all the analytes even in real complex samples as food or biological samples. The approach is very simple and can be used for the routine analysis of POP´s in food and biological samples.

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FIGURE CAPTIONS

Figure 1. HF-LPME assembly designed for the extraction of POPs. The inset shown the Eppendorf GELoader pipette tip used.

Figure 2. Chromatogram obtained from a standard solution at 10 µg L^{-1} using HF membrane extraction and analysis by GC-ECD.

Figure 3. Effect of the extraction solvents used for HF-LPME in the relative peak area of (a) Pesticides, (b) PCB's and (c) PBDE's. The error bars indicate the standard deviation (n=3). Relative peak area = peak area/lowest peak area using isooctane.

Figure 4. Effect of the stirring velocity used for the HF-LPME in the relative peak area of (a) Pesticides, (b) PCB's and (c) and PBDE's. The error bars indicate the standard deviation (n=3). Relative peak area = peak area/first peak area corresponding to the lowest experimental value of the abscissa.

Figure 5. Optimization of the volume of methanol as modifier (% v/v) used for the HF-LPME in the relative peak area of (a) Pesticides, (b) PCB's and (c) PBDE's. The error bars indicated the standard deviation (n=3). Relative peak area = peak area/first peak area corresponding to the lowest experimental value of the abscissa.

Figure 6. Optimization of the volume of acetonitrile as modifier (% v/v) used for the HF-LPME in the relative peak area of (a) Pesticides, (b) PCB's and (c) PBDE's. The error bars indicated the standard deviation (n=3). Relative peak area = peak area/first peak area corresponding to the lowest experimental value of the abscissa.

Figure 7. COMBINED 3D surfaces representing the combined response against the optimised variables.

Figure 8. Contour plot of the combined response showing the optimum.

360 **EQUATIONS**

361

362 Equation I

363

364 $$y = \beta_0 + \sum \beta_i \chi_i + \sum \beta_{ii} \chi_i^2 + \sum \beta_{ij} \chi_i \chi_j$$

365

366 Equation II

367

368 $$t_k = \frac{|b_k|}{s(b_k)}$$

369

370 Equation III

371

372 $$COMB = \sum_i \left\{ k\, w_i \left| \frac{(Y_i - G_i)}{R_i} \right| \right\}$$

373

374 Equation IV

375

376 $Ef = 1 / (V_a / V_d + 1 / K_{a/d})$; $K_{a/d} = C_{eq,a} / C_{eq,d}$

377

378