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In-drop derivatisation liquid-phase microextraction assisted by ion-pairing transfer for the gas chromatographic determination of phenolic endocrine disruptors

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Abstract

A novel in-drop derivatisation liquid-phase microextraction procedure with an ion-pairing agent is developed and optimised for the extraction of endocrine-disrupting chemicals. The ethyl esters of the analytes were rapidly formed in the organic drop and analysed by gas chromatography. The effects of various parameters such as rate and time of agitation, ion-pairing agent and reactant concentration, pH and temperature were studied systematically to optimise the process and bring out the locale of reaction in the organic drop. A study of the mechanistic pathways of the overall procedure is attempted leading to interesting findings and delineating important points of the kinetics and mechanism. A mechanistic model is proposed on the basis of the theory of mass transfer with chemical reaction in two liquid phases. The *O*-ethoxycarbonyl derivatisation appears to take place in the bulk organic phase. The system provides insight into the first reported analytical case of single-drop extraction–preconcentration–derivatisation assisted by an ion-pairing transfer and has all of the interesting facets of chemical reaction in which the role of mass transfer comes into picture.

The analytical features of the method are acceptable and the overall relative standard deviations of the intra-day repeatability ($n = 5$) and inter-day reproducibility were <3.9% and <5.4%, respectively, for gas chromatography–mass spectrometry analyses and <4.3% and <7.1% for gas chromatography–flame ionisation detection analyses. The method was applicable to urine and surface water samples. The LODs ranged between 0.2–1.3 ng mL^{-1} and 8.5–26.5 ng mL^{-1} for GC/MS and GC/FID analyses, respectively.

Keywords: Single-drop liquid-phase microextraction; Ion-pairing transfer; Ethyl chloroformate derivatisation; Endocrine disruptors

1. Introduction

An increasing number of anthropogenic chemicals have been found in the environment, generating awareness regarding their potential to disrupt the normal functions of the endocrine system of animals [1]. Collectively known as endocrine-disrupting chemicals (EDCs), they encompass steroid estrogens, which include natural and synthetic ones, polychlorinated biphenyls, chlorinated insecticides, alkylphenols, bisphenol-A, etc., which are known as xenoestrogens [2].

EDCs may cause birth defects, alter immune functions, contribute to sexual dysfunction, or can even cause cancers and heart disease in living species including humans [3,4]. These impacts may be cumulative and irreversible, appearing in following generations, endangering the sustainable development of the ecosystem [5,6]. Most of these compounds such as bisphenol-A and alkylphenols, have been detected in wastewater, sewage and groundwater and are thought to be non-biodegradable and effective EDCs [7–9]. On account of this, the European Union and the USEPA have ordered further evaluation for their endocrine disruption role of a "priority" list.

Method-development strategies usually discount analytical derivatisation at the outset because of additional steps, excess of reagent and the concomitant potential for interferences. However, there are numerous examples where analytical derivatisations are required to enhance sensitivity, selectivity, extraction efficiency and overall quality of the data. Improvements resulting from derivatisation in instrumental methods are well known [10]. The development of automated and/or miniaturised techniques in connection with the measuring analytical devices at hand, demonstrated that the concerns regarding extra steps and time requirements are not necessarily at issue.

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The literature abounds with sample preparation techniques in various guises such as liquid–liquid extraction [11], solid-phase extraction [12] and molecularly imprinted solid-phase extraction [13]. Additionally, solventless and solvent-minimised polymer sorption techniques, such as solid-phase microextraction (SPME) [14] and stir bar sorptive extraction (SBSE) [15] have appeared, exploiting the high affinity of polymeric materials towards target substances. Hollow-fibre liquid-phase microextraction (LPME) a low-cost, quasi-solventless technique has been successfully employed for the determination of EDCs in water samples [16,17]. The single-drop LPME can be regarded as an alternative microextraction technique capitalising on most of the advantages of LPME already mentioned; it uses a single organic droplet where the extraction of the analytes takes place [18,19]. A derivatisation step prior or subsequent to single-drop LPME is frequently required in order for the analytes to be analysed via gas chromatography [20,21].

For the analysis of EDCs, liquid chromatography with fluorescence [22,23], electrochemical [24,25], mass spectrometry detection [7,26,27], capillary electrophoresis with chemiluminescence detection [28] and gas chromatography–mass spectrometry (GC–MS) have been employed [29]. To block all active protons present in the phenolic compounds, the GC analytical methods proposed in the literature apply derivatisation procedures [30]. Different reagents have been used to derivatise phenolic EDCs, including pentafluorobenzyl bromide, *N*,*O*-bis(trimethylsilyl) trifluoroacetamide or *N*-(*tert*-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide that lead to the formation of pentafluorobenzyl ether [31], trimethylsilyl and *tert*-butyldimethylsilyl derivatives [31,32], respectively.

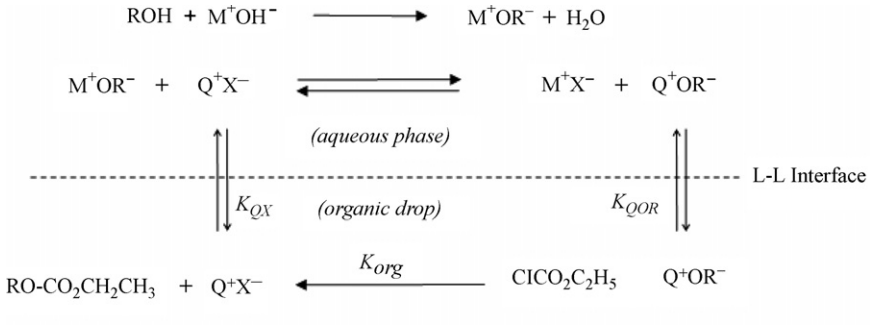
In the study herein, a one-step single-drop microextraction–preconcentration process is reported for the first time, via a rapid transfer of organic ions as ion-pairs, followed by in-drop derivatisation reaction, under mild conditions, within the bulk of the organic drop. Kramer and Andrews have applied hollow fibre-protected LPME with in-tube derivatisation and ion-pairing agent of an acidic drug [33]. However, this drop-based analysis system has got unique attributes and can constitute, a significant alternative mode to the well-known on-fibre SPME. A theoretical background is established by way of EDCs analysis in order to reveal the implicit process characteristics and some mechanistic pathways of the single-drop phase ion-pair transfer derivatisation. Gas chromatographic analysis testified to the accuracy, precision and applicability to real samples.

2. Theory

In a two-phase (aqueous–organic) system either of the phases can be dispersed into the other in droplet size, by agitation. So, the contact area of two phases can be increased with higher agitation rate. In single-drop LPME, the organic droplet macroscopically can be regarded as the dispersed phase. The single drop is viewed as a rigid analytical system; therefore, the moderate agitation applied, cannot heighten the interfacial area which can lead to enhancement of mass-transfer rates. From the analytical and theoretical point of view, it is important to quantitatively describe the diffusion-reaction behaviour in a *single*-dispersed drop which bears a reactive species, in order to assess the overall performance.

Succinctly, in the chemical system under consideration, phenolic compounds (ROH) are converted *in situ*, into the corresponding phenolates OR^-, in alkaline conditions. The produced ion-pair (Q^+OR^-) can cross the liquid–liquid interface due to the lipophilicity of Q^+, diffusing from the interface into the organic phase, this being a "phase-transfer" step. Next, it reacts with ethyl chloroformate ($ClCO_2C_2H_5$) to produce the *O*-ethoxycarbonyl derivative $RO–CO_2CH_2CH_3$ in the organic phase and the free ion Q^+ is then transferred across the interface to the aqueous phase. In the above sense, the overall process can be featured as a phase-transfer catalysis (PTC) making full allowance for the previously reported limitations due to the presence of the drop. The separate stages can be discerned and the schematic representation of the liquid–liquid ion-pair transfer-substitution reaction is shown in Fig. 1, with each step being able to be the rate-determining for the transfer and reaction process.

In the two-phase reaction, mass-transfer resistance is an important factor affecting the reaction rate and yield. Film theory postulates steady-state diffusion (after a short lag time) across stagnant solvent layers-films δ_{org} and δ_{aq} adjacent to the interface in the organic and aqueous phase, respectively [34]. At the same time, uniform, instantaneous and complete convective mixing exists in the bulk aqueous phase in a distance δ cm away from the



Q^+: quaternary ammonium ion, K: distribution constant for the ion-pair, K_{org}: Intrinsic reaction rate constant, OR^-: ionized phenolate, X^-: Cl^- or Br^-

Fig. 1. Schematic diagram of the liquid–liquid ion-pair transfer-substitution reaction.

liquid–liquid interface. This theory was proved to be an accurate model for characterizing the convective–diffusive mass-transfer properties of solvent extraction of a sample compound into a single drop [18]. However, given the occurrence of reaction, the intrinsic rate of reaction is determined by the diffusion of one of the reactants, originally resident in the aqueous phase into the single-dispersed drop where reaction and diffusion proceed simultaneously. The theory of mass transfer accompanied by chemical reaction in multiphase systems has been described by Doraiswamy and Sharma who classified these reactions into four regimes on the basis of a two-film model [35]: (1) very slow reaction in bulk organic phase, (2) slow reaction in bulk organic phase but no reaction in the organic-phase film, (3) fast reaction in the organic-phase film, and (4) instantaneous reaction of reactants diffusing at a reaction plane in organic-phase film. The theory suggests that the mass-transfer rates are prominent in the regimes (2) and (4), and thus the rate of agitation plays a dominant role.

3. Experimental

3.1. Reagents and chemicals

Riedel-de Haën (Seelze, Germany) supplied the GC grade solvents dichloromethane, chloroform, ethyl acetate, *n*-octanol, isooctane, toluene, *tert*-butyl methyl ether, diethyl ether and *n*-hexane. Ion-pairing agents [tetrabutylammonium bromide (TBAB), tetrahexylammonium bromide (THAB), cetyltrimethylammonium bromide (CTAB)], *n*-pentadecane (internal standard, IS), sodium chloride, sodium hydroxide, disodium hydrogen phosphate, bisphenol-A, 4-*n*-propylphenol, 4-*n*-octylphenol, 4-*tert*-butylphenol, 4-*n*-heptylphenol, pentachlorophenol, 2,4-dichlorophenol and β-estradiol were all obtained from Sigma–Aldrich (St. Louis, MO, USA). Ethyl chloroformate (ECF), methyl-4-hydroxybenzoate (methylparaben) and Irgasan (triclosan or 5-chloro-2-(2,4-dichlorophenoxy)phenol) were obtained from Fluka (Buchs, Switzerland). All chemicals and solvents were of the highest grade available.

3.2. Solutions

Standard stock solutions of analytes (2–5 mg mL^{-1}) were prepared separately by weight, in methanol. Successive dilutions were made in double distilled water. The single-standard solutions were combined in a mixture that was aimed to contain the desired concentrations of every analyte. The extraction solvent ($CHCl_3$/*n*-octanol, 1:1) contained *n*-pentadecane at concentration of 50 μM and ECF at 0.1 M. Disodium hydrogen phosphate–NaOH buffer solution (0.5 M) was employed for pH adjustment. The ion-pairing agents were prepared at a concentration of 0.05 M in phosphate buffer solution and were stable for several days. These solutions were prepared weekly. All glassware was cleaned with AP-13 Extran alkaline soap (Merck-Darmstadt, Germany) for 24 h, rinsed consecutively with double distilled water and acetone and baked at 110 °C, overnight. Volumetric flasks were washed as described above but were air-dried instead of baked. Single-drop experiments and injections were performed using a 10-μL microsyringe with angle-cut needle tip (0.6 mm glass barrel, i.d.; 0.11 mm needle i.d.).

3.3. Instrumentation-chromatographic analysis

The gas chromatographic system used was a Shimadzu GC-2010 gas chromatograph equipped with a flame ionisation detector (FID) and a Supelco 30 m × 0.25 mm i.d. fused-silica capillary column (SPB-5, film thickness 0.25 μm). Helium was used as the carrier gas and the flow rate was set at 1 mL min^{-1}. Samples were injected in the splitless mode with subsequent opening of vent valve after 1 min. The GC oven temperature was programmed as: 60 °C; ramp to 170 °C at a rate of 8 °C min^{-1}; ramp to 270 °C at a rate of 12 °C min^{-1}, held for 12.95 min. The total program run was 35 min. The injector and detector temperatures were set at 250 and 280 °C, respectively. Data was collected and integrated with a personal computer using the GC Solution Version 2.21.00 Chromatography Software (Shimadzu Chem. Lab. Solutions).

The GC–MS analysis of the target compounds was performed on a Shimadzu GC-17A gas chromatograph interfaced with a Shimadzu QP 5000 mass spectrometer, in the selective ion monitoring (SIM) mode. Chromatography was conducted on the same chromatographic column and under the chromatographic conditions reported above. The mass detector was operated in the electron impact (EI) mode at 70 eV and electron multiplier voltage of 1.25 kV. The mass fragments of the derivatives were obtained in the full scan mode in the scan range from *m*/*z* 50 to 650. A solvent delay time of 14.5 min was used to protect the ion multiplier of the MS instrument from saturation. System control and data acquisition were achieved with a personal computer using the CLASS-5000 Version 1.24 Chromatography Software (Shimadzu Chem. Lab. Analysis System and Software).

3.4. Analytical procedure

In a screw-capped vial of 5 mL sealed with PTFE-lined silicon septa are placed 3 mL of the sample to be analysed, 0.5 mL of buffer solution and 0.5 mL ion-pairing agent solution. The sample solution is agitated at 250 rpm with a stir bar (10 mm × 3 mm). The microsyringe is rinsed with the organic solvent for several times to ensure that no air bubbles are left in the barrel and the needle and then 3 μL of the organic solvent containing *n*-pentadecane as IS and ECF is drawn into the syringe. With the needle tip out of the solution, the plunger is depressed by 1 μL. The needle, fixed with a stand and clamps, is then immersed in the sample and the plunger is depressed to expose a 2-μL organic drop to the stirred aqueous solution for a set period of time. The drop is then retracted into the microsyringe, which in turn is removed from the sample vial and the organic solvent drop is injected into the GC system for analysis.

3.5. Sample treatment

Urine samples were collected from laboratory personnel. Lake sediment and surface water were collected from

lake Pamvotis (Ioannina, Greece) and wastewater sample was received from the sewage treatment plant of the city of Ioannina. Pore water was squeezed from lake sediment using a nylon squeezer under a nitrogen pressure of 0.3–0.4 MPa through a 0.45-μm membrane filter and analysed without further pretreatment. All samples were filtered prior to analysis and stored refrigerated, until analysis.

4. Results and discussion

4.1. Confirmation of the derivatives

The *O*-ethoxycarbonyl derivatisation is known to be applicable virtually to a multitude of compounds which contain amino-, thiol-, imidazole- or phenolic hydroxyl-groups, under different reaction conditions [36]. The molecular ion peaks of most phenols as *O*-EOC derivatives were recognizable or quite prominent (≥10% relative abundance). Almost invariably, the mass spectral patterns of expected ECF derivatives exhibit characteristic mass fragments like *m/z* $[M-44]^+$ and *m/z* $[M-72]^+$ ions corresponding to loss of EtO– group or CO_2 and elimination of EtOCO–, respectively (Table 1). The $[M-72]^+$ ions are identical to the molecular ions of their corresponding underivatised phenols except for the di-EOC derivative of bisphenol-A with two phenolic groups. *t*-Butylphenol gave the alkyl-braking fragments $[MW-15]^+$ and $[MW-29]^+$ and methyl-4-hydroxybenzoate the detachment of MeO– (*m/z* 193) and of MeOCO– (*m/z* 165). The intense fragment ions of *m/z* 107 and *m/z* 135 correspond to $[CH_2C_6H_4OH]^+$ and $[C_3H_6C_6H_4OH]^+$, respectively. All di-hydroxyl phenols as bearing two active hydrogens, resulted in di- and mono-substituted derivatives, the latter being in negligible yield. The base peak ion of *m/z* 213 for bisphenol-A was assumed to be formed by the elimination of CH_3, $CO_2CH_2CH_2$ and $CO_2CH_2CH_2$ from its molecular ion. β-Estradiol was the analyte that did not present characteristic mass fragmentation exhibiting only the molecular ion of the underivatised compound. In order to ratify derivatisation of β-estradiol, standard solution of the compound in chloroform was injected into GC/MS. The retention time of β-estradiol does not coincide with the adduct after derivatisation reaction, inferring derivatisation of β-estradiol albeit no definite conclusion can be drawn as to whether mono- or di-substituted derivative is generated since the fragmentation pattern does not facilitate structural identification of the formed adduct.

4.2. Optimisation of the extraction-reaction conditions

The dynamic characteristics of the microextraction process are closely related to the mass transfer of the analytes from the aqueous to the organic phase, mainly driven by the difference-gradient of concentration of the ion-pair between aqueous and organic phases. The experimental parameters for the extraction–derivatisation of the 10 analytes were optimised by a two-stage sequence of variations. After the first optimisation, all parameters were varied again one-at-a-time, defining a second set of values. The main criterion for the experiment was to maximise the derivatisation product yields for the 10 analytes in a reasonable time period. The chromatographic peak height ratio (analyte-to-IS) was used to evaluate the extraction efficiency under different experimental conditions.

4.2.1. Extraction solvent

The extraction solvent holds an important bearing on the transfer of analytes and the formation of derivatives. Dichloromethane, chloroform, ethyl acetate, *n*-octanol, isooctane, toluene, *tert*-butyl methyl ether, diethyl ether and *n*-hexane were tested covering a wide range of polarity, density and water-solubility. The best results with respect to recovery of analytes

Table 1
Characteristic ions of the major chromatographic peaks of the derivatised analytes

Analyte	MW of the derivative formed	$[M]^+$	$[M-44]^+$	$[M-72]^+$	Charactiristic ions	Other ions
2,4-Dichlorophenol	235	234	191	<u>163</u>		207, <u>148</u>, 189, 133
4-*n*-Propylphenol	208	208	164	<u>136</u>		<u>107</u>, 93
4-*tert*-Butylphenol	222	222	178	150	MW-15 = 207 MW-29 = 193	207, 193, <u>163</u>, <u>135</u>, 107
Methyl-4-hydroxybenzoate	224	224	180	<u>152</u>	MW-31 = 193 MW-59 = 165	137, <u>121</u>
Pentachlorophenol	338	338	294	<u>266</u>		230, 202, <u>251</u>
4-*n*-Heptylphenol	264	264	220			192, <u>135</u>, <u>107</u>
4-*n*-Octylphenol	278	278	234	<u>206</u>		177, 163, <u>135</u>, <u>107</u>
Irgasan	361	361	<u>317</u>	<u>289</u>		312, 281, 265, 252, 240, 236, 218, 191
Bisphenol A	372	372		300	MW-15 = 357 MW-2 × 73 = 227	313, 269, <u>285</u>, <u>241</u>, <u>213</u>
β-Estradiol						<u>272</u>, 254, 226, 213, 197, 186, 172, <u>162</u>, 146, 133

Underlined ions are used for identification and quantification purposes.

were in the order of dichloromethane > chloroform > *n*-octanol. The remainder of the solvents afforded far less recoveries. Between dichloromethane and chloroform the latter was preferred because of its lower solubility in water. However, during the process of optimisation, it appeared that chloroform droplet (2 μL of volume) tended to become heavier in the course of time, a fact that readily lead to its detachment from the needle tip. To overcome this problem, mixtures of chloroform with different organic solvents of lower density than water (e.g. *n*-octanol, ethyl acetate, toluene and hexane) were tested. Chloroform/*n*-octanol (1:1) was chosen because it provided the most reproducible results and drop stability without compromising the sensitivity of the method.

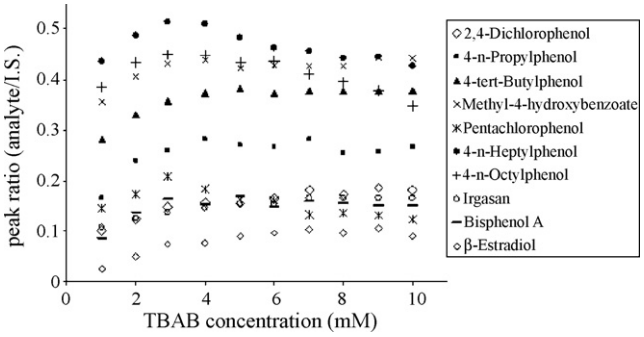


Fig. 2. Effect of TBAB ion-pairing agent on the extraction-reaction of phenolic endocrine disruptors.

4.2.2. Type and amount of ion-pairing agent

The ion-pairing agents were chosen based on their low toxicity and proven effectiveness for a wide range of reactions in the domain of analytical chemistry [37–43]. In a two-phase system (aqueous:organic, 1:1) using the same reactant concentrations, under vigorous agitation but in the absence of the ion-pair agent, either reaction does not take place or some of the analytes of concern react very slowly giving rise to low derivatisation yields. The yields of the derivative formation were even lower when single-drop LPME without ion-pairing agent was used within the limited period of time that droplet is allowed to interact. Phenolates are fairly hydrophilic and difficult to transfer to the organic phase. The addition of the ion-pairing agent leads to rapid interaction, significantly increasing the yields of the ECF adducts (at least a 20-fold improvement of recoveries was determined) in a reasonable time.

With the proper choice of Q^+ regarding its lipophilicity, maximal concentration of the anion in the organic phase, and hence maximal rate for a transfer-controlled reaction can be achieved. Optimisation of the ion-pairing type was performed by using soluble ion-pairing agents such as TBAB, THAB and CTAB. The THAB and TBAB exhibited the most favourable features. The high solubility of TBAB in both organic and aqueous phase and the electrostatic interactions led to the best recovery results for the derivatisation of the compounds under study. However, there is another point which should be made with respect to the ion-pair nature: the greater the approach of the phenolates to the cation the stronger the electrostatic interactions are between them thereby lowering the reactivity of the anions. According to Halpern, the rate of reaction of the anionic part of the ion-pair with a substrate can be estimated by a value which should optimally be <1 for higher anion reactivity [44]. This semi-empirical value is the sum of the reciprocals of the number of carbon atoms in the chains, being $4 \times 1/4 = 1$ for TBAB, which is marginally acceptable for the reaction of the ion-pair with ECF. It is therefore, reasonable that unlike the other two above mentioned ion-pair agents, the criterion for moderate electrostatic interaction between TBAB and phenolates is fulfilled.

Various TBAB concentrations were tested (1–8 mM) and it was shown that increasing TBAB concentration led to increase in extraction–derivatisation for certain analytes such as 2,4-dichlorophenol which is a characteristic of PTC [45] yet to decrease of others like 4-*n*-octylphenol and pentachlorophenol. This trend is illustrated in Fig. 2 and the associated effects could be traced to unpredictable mass-transfer phenomena and the increase in ionic strength of the solution and repulse of anions. A final TBAB concentration of 3 mM in the analysed solution was selected for maximum recovery of all analytes.

4.2.3. Stirring rate

Effective mixing of the aqueous phase is essential for the overall procedure and thus mixing should be vigorous enough so as to maintain at the surface and within the bulk of the organic drop the ion-pair by convective–diffusive transfer in the aqueous phase. At the same time, it should be sufficiently mild to maintain the droplet at the needle tip. To appraise kinetic- or diffusion-limited reaction realm it was necessary to determine the dependence of the initial reaction rate on the stirring rate. The influence of mass-transfer resistance for the transfer of reactants to the reaction phase is ascertained by varying the rate of agitation in the range of 50–300 rpm under otherwise similar conditions. Faster agitation can increase the rate of derivatisation through increasing the mass-transfer rate of phenolates to the droplet. It was observed that all 10 analytes were converted to *O*-ethoxycarbonyl derivatives in increasing yields, within shorter reaction time periods, at an agitation rate from 50 to 300 rpm. For the prevailing experimental conditions a stirring rate of 250 rpm proved to be adequate for rapid and reproducible analysis. Stirring at higher rates bring about vibrations to the hanging droplet leading to lower reproducibility and risk of detachment of the drop. From the stirring rate study, intuitively, a diffusion-limited reaction instead of a kinetically controlled one is to be elaborated subsequently.

4.2.4. Presence and amount of salt (salting-out effect)

Increased ionic strength of the aqueous solution gave also contradictory results affecting positively the derivatisation of some compounds and negatively others. Although it is accepted that increased ionic strength lead to decrease in water solubility of the analytes enhancing consequently the extraction yield, several researchers reported the opposite effect for single-drop LPME analyses [18]. The physical properties of the Nerst diffusion film may be altered by the dissolved NaCl reducing the rate of diffusion of the target analytes into the drop. Taking into

account that the decrease in the yields of reactions outweighs the less significant positive effects on the system, we opted for not adding NaCl in the analysis sample.

4.2.5. Amount of ethyl chloroformate

Ethyl chloroformate was used in excess of 0.1 M. This amount ensures as high derivatisation yield as possible and reconciles the need for an excess of derivatisation reagent which besides, decomposes under alkaline conditions.

4.2.6. pH of the analytical solution

Alkaline pH is essential for the reaction to occur as it promotes the deprotonation of the reactive species. A pH range 7–12 was studied in order to establish the optimum value for the reaction to occur. Increasing pH led to improved reaction yields reaching maximum at pH 10.5. Raising the pH further, the recoveries did not improve upon; on the contrary, they decreased significantly at pH 12, presumably due to heightened hydrophilicity of the ion-pairing agent, degradation of it or oxidative degradation of phenols [43].

4.2.7. Kinetic aspects and mechanistic considerations

The kinetic experiments conducted showed a gradual increase in extraction–derivatisation yield as a function of time (Fig. 3). Di-hydroxy analytes react at slower rates giving lower yields than their mono-hydroxyl analogues. Steric hindrance, electronic behaviour of the substituents and the number of hydroxyl groups available for derivatisation in the context of a multiple-step process are some of the determining parameters of the study. A diminution of the product yield after the elapse of 15 min where a plateau has been acquired, does not allow for monitoring the reaction thereafter. Thus, a reaction time of 12 min was selected for the next experiments. In order to confirm that there were no underivatised analytes in the drop, after the completion of the extraction–derivatisation, the drop was withdrawn in the syringe barrel and left to stand for periods of 0, 5, 10 and 15 min. From the experimental results it appeared that reaction does not progress in the drop bulk beyond the 12 min.

The effect of temperature on the rate of reaction was studied in the range of 20–35 °C, under otherwise constant reaction conditions. It was essential that the aqueous-phase temperature be kept low in order to avoid dissolution of ECF and shrinkage of droplet at elevated temperatures. On the other hand, temperatures lower than 25 °C led to slower reaction rates and poorer recoveries. Predictably, there should always be a trade-off between drop stability and efficiency. A temperature of 25 °C was eventually chosen as the optimum for the analysis. The yields of the overall conversion were found to increase marginally with increasing temperature from 20 to 35 °C, which suggested that the reaction might not be free from mass-transfer effects.

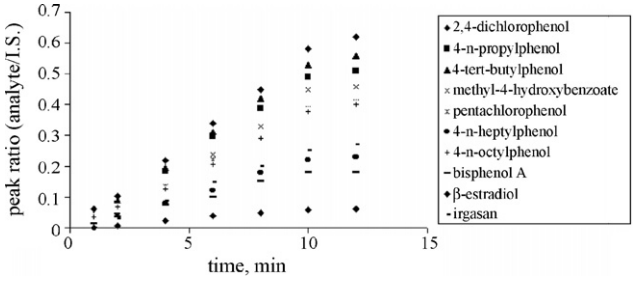


Fig. 3. Kinetics experiment for the 10 analytes under study with the proposed method of extraction–derivatisation.

The initial rates of reaction were calculated and the energy of activation (E_a) was determined from the slope of Arrhenius plot ($\ln(k_{obs})$ versus $1/T$) to estimate whether the reaction is mass-transfer limited or not. The E_a values were found to be 1.2–3.1 kcal mol^{-1} for the analytes of interest, which signifies that mass-transfer limitations are present as opposed to the faster rates of the reactions. The pronounced effect of the rate of agitation, the trivial impact of temperature on the conversions and initial rates and the low activation energies advocate reactions involving a mass-transfer effect. The reactions can take place in both phases (aqueous and organic) and E_a values are taken as a guideline to deduce a mechanism of the process. However, the ECF is practically insoluble in water and gradually decomposes by it. It is prudent to suppose that the reactions are realised in the organic drop and might be in regime 2 (slow reaction) or regime 4 (instantaneous reaction) relying on the theory of mass transfer with chemical reaction, as described in Section 2 (Theory). The case of instantaneous reaction is ruled out, as the kinetic profiles in Fig. 3 predict. Therefore, extraction–derivatisation in the organic drop should conform to the so-called regime 2, which can be considered as a typical example of normal liquid–liquid PTC [42]. In the regime considered, seemingly, the overall rate is governed by the rate of mass transfer and the reaction occurs in the bulk organic phase rather than in the stagnant diffusion film next to the interface in the organic drop phase. According to the same theory, there should be a concentration gradient for the ion-pair in the film of the organic droplet, and its concentration in the bulk reaction phase is zero. The above considerations are exemplified in Fig. 4.

4.3. Analytical method

4.3.1. Figures of merit

A series of standard composite solutions were prepared in triplicate, over the range of 0.5–800 ng mL^{-1}. The derivatives were quantified by the area ratios relative to the IS. The amounts injected and their respective response ratios (analyte-to-IS) were used for the construction of the calibration plots and quantification. Satisfactory linearity was obtained for the employed GC/MS and GC/FID method as demonstrated by correlation coefficients higher than 0.9875 and 0.9865, respectively, throughout the method validation. Linear responses were observed over the ranges given in Table 2.

The limits of detection were estimated as $3S_b$/slope of the calibration curve, where S_b is the standard deviation of the blank measurements ($n = 3$). The actual LODs were then determined by the analysis of samples of known concentrations and found to range from 0.2 to 1.3 ng mL^{-1} for the GC/MS analyses and from 8.5 to 26.5 ng mL^{-1} for GC/FID analyses. In the same

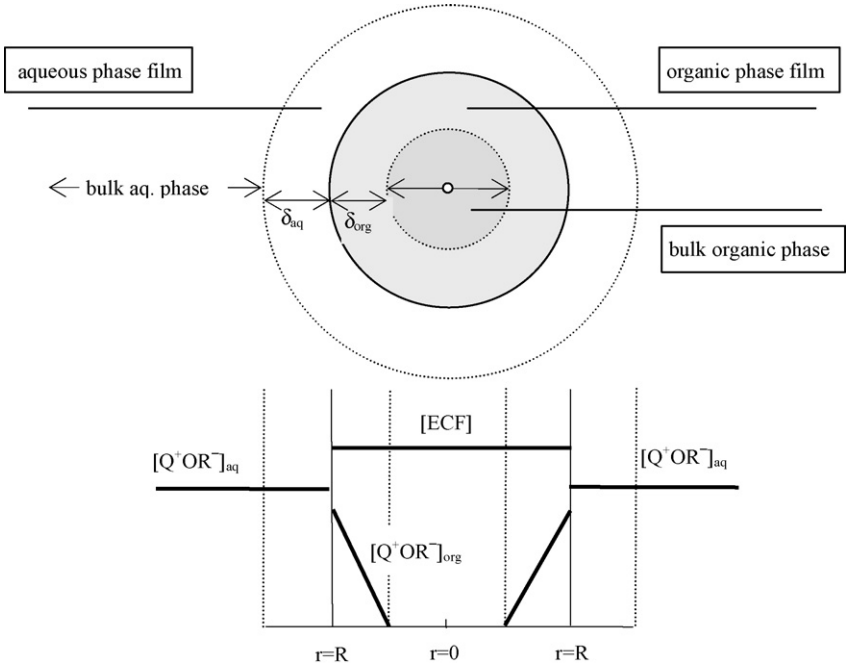


Fig. 4. Schematic sketch for the concentration profiles of the reacting species Q^+OR^- and ECF ($ClCO_2C_2H_5$) in a single-dispersed drop hinging on the relative rates of mass transfer and chemical reaction. There is no resistance to mass transfer of ion pairs Q^+X^- and Q^+OR^- on the aqueous side. Shaded areas denote the spherical organic drop. Solid dark line represents the liquid–liquid interface. Dotted line in the drop denotes the limits of liquid films (aqueous and organic).

way, the limits of quantitation were estimated as $10S_b$/slope corresponding to the lowest values of the calibration curves.

To verify the precision of the proposed method, within-day and between-day precision of processed standards in the range 1.5–100 ng mL^{-1} were obtained. The overall relative standard deviations of the intra-day repeatability ($n = 5$) and inter-day reproducibility (five consecutive days, three replicates each day) were <3.9 and <5.4%, respectively, for GC/MS analyses, and <4.3 and <7.1% for GC/FID analyses.

4.3.2. Sample analysis

The method practicability and applicability were ratified by the analysis of real samples. Urine, lake water, sediment pore water and wastewater were analysed after filtration without further pre-treatment. The optimised extraction–derivatisation protocol was applied to these samples and their analytes content was calculated from the appropriate calibration curves. Of the samples analysed only urine contained non-detectable concentrations of the disruptors. In all other cases, some of disruptors were found to be at concentrations around the GC/MS limits of detection. Fortified samples spiked at two concentration levels (i.e. three and six times the respective limits of quantitation) for the 10 compounds were analysed. The concentration of each compound was determined by interpolation from the standard calibration curve within the dynamic linear range and compared with the added amount. The recovery values ranged from 75 to 108% for the samples tested. A reasonable explanation for recoveries greater than a 100% can be the positive influence of co-extractives of the spiked urine on the chromatographic response of certain analytes. Essentially, absence of matrix inter-

Table 2
Analytical figures of merit of the method for the GC/MS (SIM) and GC–FID analyses

Analyte	GC/MS(SIM)			GC/FID		
	R^a	DLR (ng mL^{-1})[b]	LOD (ng mL^{-1})[c]	R^a	DLR (ng mL^{-1})[b]	LOD (ng mL^{-1})[c]
2,4-Dichlorophenol	0.9983	1.3–30	0.4	0.9989	46–400	15.8
4-*n*-Propylphenol	0.9960	1.5–37	0.5	0.9944	41–450	14.0
4-*tert*-Butylphenol	0.9994	0.5–27	0.2	0.9952	36–420	12.0
Methyl-4-hydroxybenzoate	0.9935	2.5–44	0.8	0.9970	42–580	14.0
Pentachlorophenol	0.9974	0.5–25	0.2	0.9987	23–580	8.5
4-*n*-Heptylphenol	0.9969	1.8–25	0.6	0.9949	39–490	12.3
4-*n*-Octylphenol	0.9986	0.8–25	0.3	0.9925	52–610	17.3
Irgasan	0.9966	1.1–28	0.4	0.9965	55–720	18.3
Bisphenol A	0.9880	2.2–43	0.7	0.9865	53–760	17.0
β-Estradiol	0.9875	3.8–51	1.3	0.9880	80–800	26.5

[a] Correlation coefficient calculated from three replicates at six concentration levels.
[b] Dynamic linear range.
[c] Limit of detection.

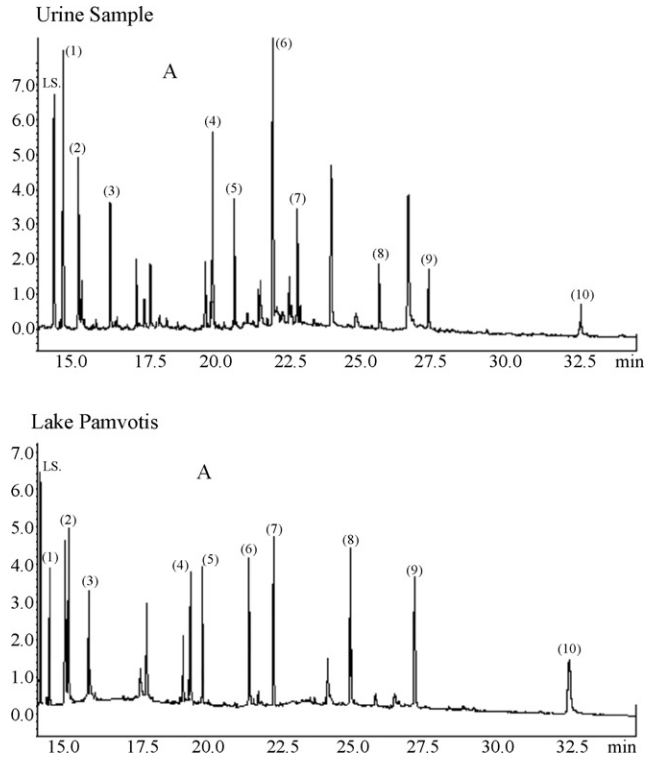


Fig. 5. GC/FID chromatographic traces of urine and lake water samples. Peak identification was confirmed by GC/MS. IS, internal standard; (1) 2,4-dichlorophenol; (2) 4-*n*-propylphenol; (3): 4-*tert*-butylphenol; (4) methyl-4-hydroxybenzoate; (5): pentachlorophenol; (6) 4-*n*-heptylphenol; (7) 4-*n*-octylphenol; (8) irgasan; (9) bisphenol-A; (10) β-estradiol.

ference was confirmed through the analysis of two different spiked samples. Chromatographic traces of spiked urine and lake water sample with flame-ionisation detection are depicted in Fig. 5.

5. Conclusions

Simultaneous extraction–preconcentration–derivatisation of 10 known phenolic endocrine disruptors is achieved applying an ion-pairing transfer procedure on single-drop liquid-phase microextraction. A two-phase ion-pairing is used to accelerate mass transfer and derivatisation of the analytes. The 10 compounds react readily under mild conditions and their respective ECF-derivatives are extracted into the organic droplet and directly injected for GC analyses.

The method is characterised by short analysis time, adequate reproducibility, but rather higher detection limits as compared to other published methods. The application of the method to real aqueous samples proved its practicability. This unique single-drop example of mass transfer with chemical reaction of analytical relevance has provided fundamental analysis and insight into the first reported analytical case of extraction–preconcentration–derivatisation in a single-drop assisted by an ion-pairing transfer. It is conceivable that other extraction–derivatisation analytical procedures can be feasible even with different kinetic and mass-transfer behaviour, which can be elaborated in like manner.

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