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Acid-Induced Phase Separation of Anionic Surfactants for the Extraction of 1,4-Dichlorobenzene from Honey Prior to Liquid Chromatography

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The acid-induced liquid−liquid phase separation of anionic surfactants in aqueous solutions and its applicability to cloud point extraction methodology were applied as a tool for the extraction of 1,4-dichlorobenzene (*p*-DCB) from aqueous samples. *p*-DCB is extracted into the micelles of sodium dodecane sulfonate (SDSA) in a 4.2 M HCl solution. The micellar phase is separated from the bulk aqueous solution after centrifugation and collected from the surface of the suspension. The micellar extracts are injected into a high-performance liquid chromatographic apparatus and quantified at 225 nm with a reference wavelength of 280 nm. Following the proposed methodology, a preconcentration factor of ca. 160 is achieved (starting from 50 mL solutions) allowing for detection limits at the low μg/L level. Application to honey samples produced detection limits of 2.5 μg/kg with quantification limits of 7.5 μg/kg, while the recoveries of the method ranged from 85% at high concentrations to 95% at lower concentrations of *p*-DCB. The combined uncertainty of the entire analytical procedure was 4.5% at the concentration level of 30 μg/kg allowing for reliable and reproducible results for the determination of *p*-DCB at the concentration levels considered as thresholds for EU and U.S. legislation (10 μg/kg).

KEYWORDS: 1,4-Dichlorobenzene; anionic surfactants; phase separation; honey; HPLC-UV

INTRODUCTION

1,4-Dichlorobenzene or *para*-dichlorobenzene (*p*-DCB) is a chlorinated aromatic compound of low molecular mass. It is not a natural product but is manufactured through chlorination of benzene. It has a distinct odor even at low concentrations, and because of this property, it is used regularly as a deodorant in lavatories and interior spaces (*1*). Its main household use is for the protection of woolen clothes from moths. As a moth repellent, *p*-DCB is extensively used in apiculture to protect the honey bee combs from damage induced by *Galleria mellonella*, commonly known as the wax moth (*1*, *2*). The aforementioned use has recently aroused considerable dispute as *p*-DCB has been detected at high concentrations in honey products. This alert led to a series of reactions affecting the honey market, dictating the need to protect consumers from *p*-DCB-polluted honey and therefore to develop methodologies for its accurate, reliable, and reproducible determination even at low μg/kg levels.

According to the International Agency for Research on Cancer, *p*-DCB is classified in group 2B of substances under inspection for possible carcinogenic activity (*3*). The U.S. Environmental Protection Agency has also recognized *p*-DCB as a class C—possible human carcinogen—setting a maximum contaminant level of 75 μg *p*-DCB per liter of environmental water (*4*). For drinking water, the threshold has been set even lower by some authorities to 6 μg/L, while for surface and irrigation water, 10 μg/L is the maximum allowed value (*5*). For honey and food in general, there has been no limit set by law neither in the United States nor in the European Union. Switzerland has established a maximum residue limit of 10 μg/kg (*6*). This limit has recently been proposed for adoption by Greece and consequently the European Union.

Although the control of *p*-DCB's residues in honey has been made imperative, because of its potential health hazards, the difficulty in removing it from beeswax, and the recent alert of its high abundance—as it had been used legally for a long period of time in most honey-exporting countries—the number of publications related to *p*-DCB is disproportionaly limited, in contrast with the large number of publications devoted to the monitoring of antibiotics and acaricides. Only recently, Tananaki et al. (*7*) have reported on a method for the accurate analysis of down to 10 μg/kg of *p*-DCB in honey using a purge-trap and gas chromatography−mass spectrometry (GC-MS) system. In general, the most popular methods rely on GC with a mass selective detector for peak identification, while electron capture detectors are also popular due to their increased sensitivity toward halogenated compounds (*7*−*12*).

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The use of anionic surfactants to extract hydrophobic species via so-called acid-induced phase separation was introduced several years ago by Casero et al. (*13*). Anionic surfactants are forced to form micelles in a highly acidic environment. These micelles—bearing a nonpolar core and a polar surface—along with the entrapped molecules are separated from the bulk aqueous phase by centrifugation, forming a surfactant rich phase, which in this case is driven to the top of the aqueous layer. This approach has been successfully applied to a plethora of substrates in order to extract a variety of analytes, often producing recoveries close to 100% (*13*−*15*).

In this work, we take advantage of the aforementioned feature applying the anionic surfactant cloud point phenomenon in order to extract *p*-DCB from aqueous honey mixtures. The surfactant rich phase is collected from the surface of the mixture after proper centrifugation. The viscosity of the micelle is reduced with the addition of acetonitrile. In this way, a preconcentration factor of 160 is achieved and *p*-DCB can be easily quantified at the μg/L level with a UV detector connected to an high-performance liquid chromatography (HPLC) unit. Recoveries from aqueous samples are almost quantitative, while from the honey samples, recoveries range from 85% at high concentrations to 95% at low concentrations.

EXPERIMENTAL PROCEDURES

Apparatus and Software. The liquid chromatograph consisted of a Shimadzu 10AD series for HPLC equipped with a UV−visible variable wavelength detector (Shimadzu) set at 225 nm while the reference wavelength was set at 280 nm. A LiChrospher 100 RP-18 (244 mm × 4.4 mm i.d., 5 μm) column linked to a LiChrospher guard precolumn (10 mm × 4.6 mm i.d.) and thermostated at 40 °C in a CTO-10A Shimadzu column oven was used for all separations. Data collection and manipulation were performed by means of a CLASS-VP Shimadzu automated software for chromatography. A Vortex Velp Scientifica mixer was used for thorough mixing of solutions. A Sorvall RC-5B refrigerated superspeed centrifuge (Du Pont Instruments) was used for phase separations. An Ultrasonic thermostated bath (Bandelin Sonorex RK 100, Germany) maintained at the desired temperatures was also used for extraction experiments.

Reagents. *p*-DCB was of analytical grade and was purchased from Sigma Aldrich Chemical Co. (WI). Appropriate amounts were diluted with methanol to prepare 100 mg/L stock solutions, which were further diluted with ultrapure water to prepare working solutions (10−1000 μg/L). Thus, the methanol content, which usually hampers clouding, was less than 1%. All solvents used were HPLC grade. Sodium dodecane sulfonic acid (SDSA) from Fluka was used without further purification (purity ≥ 97%, 2% H_2O).

Cloud Point Extraction from Aqueous Samples. Thirty-five milliliters of aqueous solution containing various concentrations of *p*-DCB, 15 mL of concentrated 12 M HCl, and 0.1 g of SDSA was added to a 50 mL centrifugal tube. The mixture was stirred vigorously for 5 min in a Vortex, and then, it was allowed to stand for 30 min in a water bath set at 65 °C. Phase separation was achieved by centrifugation at 4000 rpm (ca. 3500*g*) for 20 min. From the surfactant rich phase accumulated at the surface of the solution (ca. 250 μL), 100 μL was withdrawn with the aid of a micropipet and was subsequently diluted with 30 μL of acetonitrile to reduce its viscosity. Twenty microliters of this solution was injected in the liquid chromatograph.

Cloud Point Extraction from Honey Samples. A 10.0 ± 0.5 g amount of honey, 15 mL of 12 M HCl, 0.05 g of SDSA, and 1.0 g of NaCl were added to a 50 mL centrifugal tube, and the mixture was diluted to 50 mL with ultrapure water. The mixture was stirred vigorously with the aid of a Vortex, and then, it was transferred to a thermostated ultrasonic bath set at 65 °C and 400 W ultrasonic power for 30 min. The ultrasounds were turned off, and the tube remained at 65 °C for another 1 h in order for effective micelle formation. Finally, the mixture was centrifuged for 20 min at 4000 rpm (ca. 3500*g*) in order for phase separation to be achieved. A 150 μL amount was withdrawn from the surfactant rich phase and was again spiked with 50 μL of acetonitrile before 20 μL was injected into the HPLC unit.

Chromatographic Conditions. The elution of *p*-DCB from the micellar extract was performed with a mixture of acetonitrile and water according to the following chromatographic pattern adopted previously for the separation of 16 polycyclic aromatic hydrocarbons (*16*): The initial composition of the eluent was 47−53% H_2O−ACN, which was turned to 12−88% H_2O−ACN in 20 min and to 0−100% H_2O−ACN in another 12 min. This composition was retained for 13 min assuming the primary values (47−53% H_2O−ACN) after 10 min. A flow rate of 0.5 mL min^{-1} was adopted throughout the chromatographic run. The detection and quantification of *p*-DCB were performed by measuring the absorption at 225 nm with a simultaneous monitoring at 280 nm.

Table 1. Analytical Features of the Proposed Methodology[a]

parameter	aqueous solution	honey samples (10 g)
volume phase ratio	0.0062	
preconcentration factor	160	
linear range (μg/L)	1.5–800	
LOD	0.5 (μg/L)	2.5 (μg/kg)
LOQ	1.5 (μg/L)	7.5 (μg/kg)
RSD (%)	1.1	4.5
regression equation	$E = (150 \pm 1) \times C$ (μg/L) $+ (5 \pm 4)$	
correlation coefficient (r^2)	0.9990	

[a] LOD, limit of detection calculated as three times the signal-to-noise ratio; LOQ, limit of quantitation calculated as 10 times the signal-to-noise ratio; and RSD, relative standard deviation determined at the 30 μg/kg level (6 μg/L).

Table 2. Recovery and Preconcentration Factors after Anionic Surfactant CPE of *p*-DCB from Aqueous Matrixes

p-DCB (μg/L)	found (μg/L)	recovery (%)	preconcentration factor	theoretical preconcentration factor
3	2.9 ± 0.1	96.7 ± 3.3	158 ± 3	160
10	10.2 ± 0.1	102.0 ± 1.0	162 ± 1	160
50	49.8 ± 0.5	99.6 ± 1.0	160 ± 1	160

Table 3. Summary of Multiple Regression and ANOVA Results for *p*-DCB Recovery from Honey Following the Proposed Methodology

term	coefficient	sum of squares	degrees of freedom	*p* value
intercept	86.2			
temperature	10.9		1	0.000000015
time	2.9		1	0.0024
NaCl	4.1		1	0.00016
temperature^2	−2.9		1	0.00079
NaCl^2	−2.0		1	0.0088
regression		2555.1	5	
linear		2332.7	3	
aquare		222.4	2	
residual		321.2	11	
total			16	
$R = 0.98$	$R^2 = 0.96$			

RESULTS AND DISCUSSION

The phase separation behavior of SDSA in aqueous HCl solutions has been well-documented in several papers over the past decade (*13*−*15*). The optimum experimental conditions (surfactant amount, phase volume ratio, surfactant rich phase volume, etc.) required in order to obtain the maximum recoveries and preconcentration factors have therefore been adopted by the literature. Subsequently, an experimental design was set forth in order to produce a calibration curve and to evaluate the

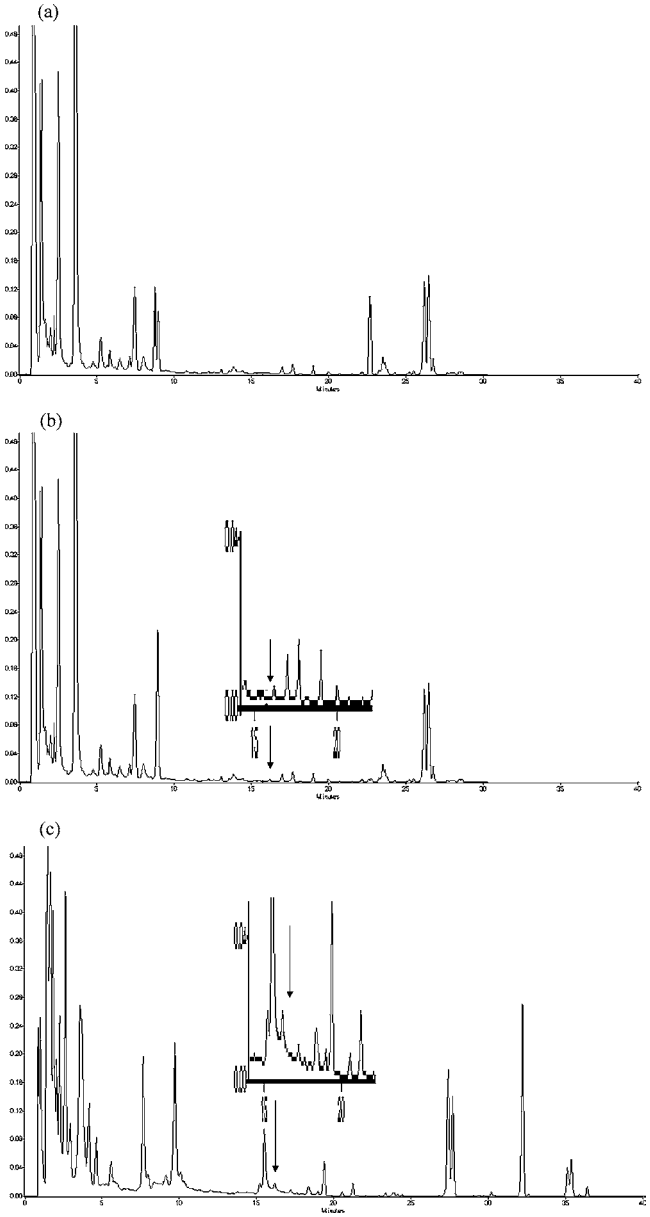

Figure 1. Chromatograms of (**a**) a *p*-DCB free honey extract, (**b**) a honey extract spiked with 10 μg/kg of *p*-DCB, and (**c**) a honey extract spiked with 30 μg/kg of *p*-DCB obtained after anionic surfactant cloud point extraction according to the proposed methodology. Inserted graphs show magnifications of the area near the *p*-DCB peak. Arrows point at *p*-DCB.

analytical characteristics of the method. The obtained features are depicted in **Table 1**. It is obvious that *p*-DCB can be satisfactorily determined at the low μg/L level starting from aqueous solutions. Under these conditions, recovery experiments following the cloud point methodology were performed at three concentration levels in order to assess the recovery of *p*-DCB from aqueous matrices and to estimate whether the achieved preconcentration factors are similar regardless of the original amount of *p*-DCB. As depicted in **Table 2**, it is obvious that the recovery is quantitative (96.7−99.6) at all concentration levels, producing preconcentration factors very close to the expected theoretical ones (158−162); therefore, the method produces accurate results for pure aqueous solutions.

The significant parameter of this work is to evaluate the ability of anionic surfactant micelles to extract *p*-DCB from a complex matrix such as honey. For this reason, a *p*-DCB-free honey was spiked with *p*-DCB in order to give a final concentration of 30 μg/kg and several parameters were examined in order to optimize the obtained results in terms of recovery.

Table 4. Recovery Experiments of *p*-DCB from Honey Following the Proposed Method

μg/kg honey		
p-DCB added	*p*-DCB determined	% recovery
10.0	10.5 ± 1.0	105 ± 10
30.0	28.5 ± 1.7	95 ± 5.7
50.0	46.5 ± 2.0	93 ± 4.0
80.0	73.5 ± 2.5	92 ± 3.1
100.0	90.8 ± 2.8	91± 2.8
200.0	170.6 ± 4.8	85 ± 2.4

Table 5. Results from Applying the Proposed Method to Greek Honeys

sample no.	*p*-DCB (μg/kg)[a]	sample no.	*p*-DCB (μg/kg)[a]
1	<LOQ	6	120 ± 8
2	<LOQ	7	8 ± 1
3	<LOD	8	<LOQ
4	38 ± 3	9	<LOQ
5	<LOQ	10	<LOD

[a] Results of five extractions and duplicate runs. ± values correspond to maximum and minimum concentrations found for the analysis of the same sample. The concentration in honey was determined according to the formula: $C_{honey} = C_{extract}$ (μg/L) × V_{dil} (L)/m_{honey} (kg).

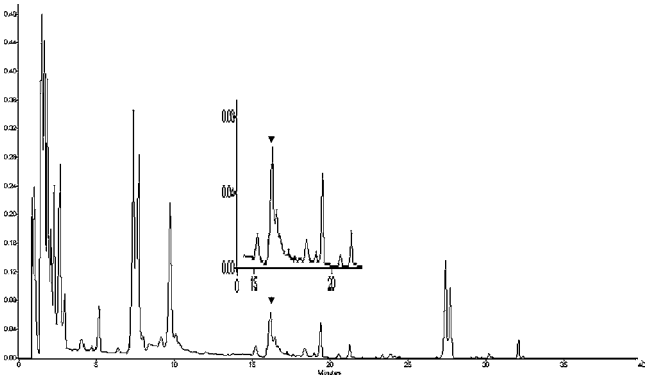

Figure 2. Chromatogram of a honey sample containing *p*-DCB well over the legislation limit obtained after anionic surfactant cloud point extraction according to the proposed methodology. The inserted graph shows magnification of the area near the *p*-DCB peak. The arrow points at *p*-DCB.

Multivariate Analysis of the Critical Parameters. Preliminary experiments showed that there are three critical parameters, that is temperature, time, and amount of added NaCl, controlling the extraction efficiency of *p*-DCB from honey samples. To assess the extent of their influence and to examine their possible interactions among each other, a central composite experimental design was set forth including 17 sets of combinations of the aforementioned variables (14 and three repetitions of the central point). The dependent variable was the recovery achieved for a 30 μg/kg *p*-DCB sample. From the multiple regression and analysis of variance (ANOVA) analysis of the obtained results, it became obvious that each individual parameter along with the squared terms of temperature and NaCl concentration is significant ($P < 0.05$) for the recovery of *p*-DCB, while the positive sign of the respective coefficients denotes that all variables have a promoting effect on the obtained signal producing an almost linear regression ($R^2 = 0.96$); therefore, there is no point of curvature. On the other hand, it was found that the interactions among the selected variables are not

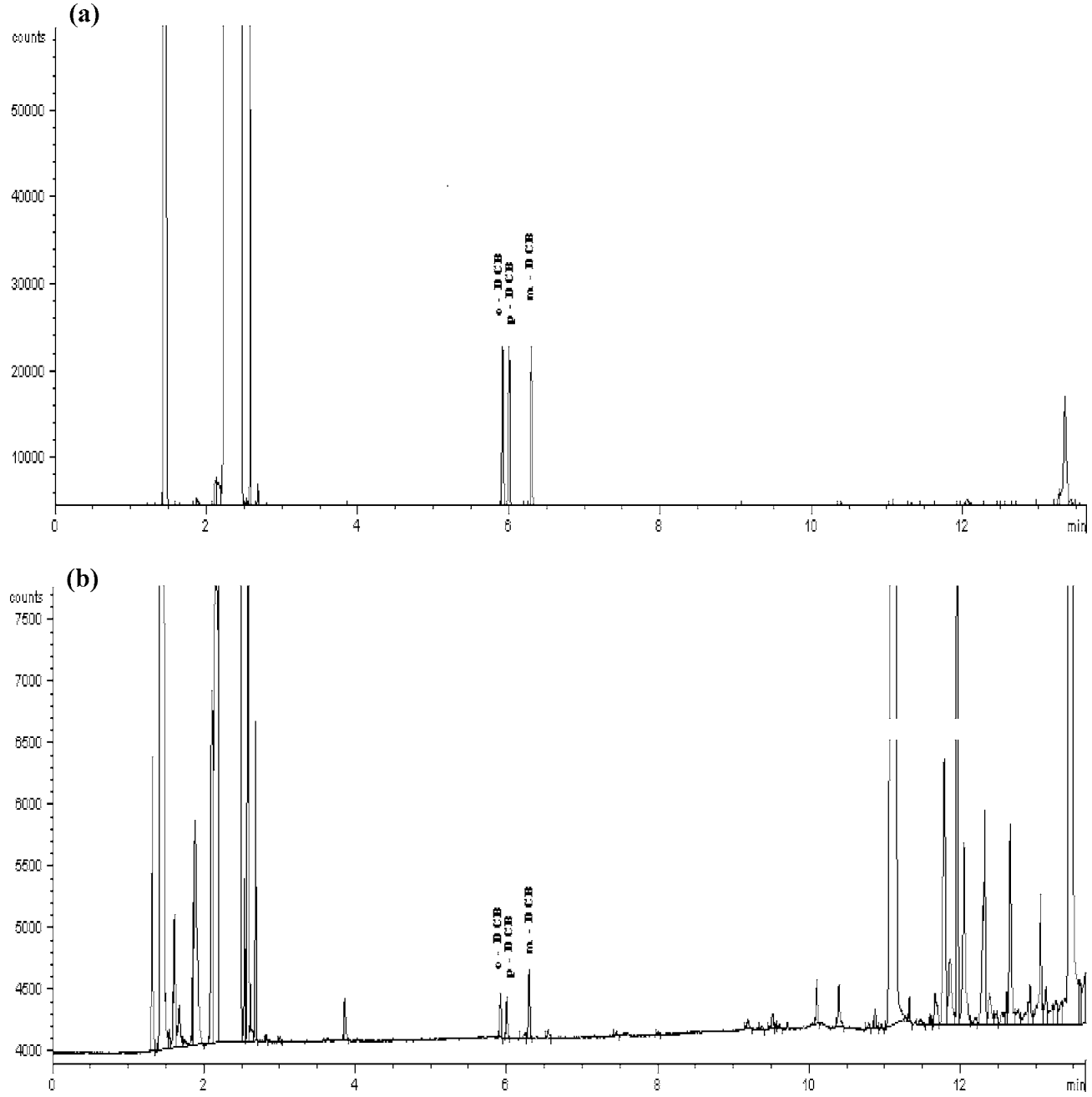

Figure 3. Gas chromatograms of (**a**) an aqueous and (**b**) a honey extract spiked with all dichlorobenzene isomers, i.e., *ortho*-, *para*-, and *meta*-dichlorobenzene, obtained after anionic surfactant cloud point extraction and back-extraction into isooctane.

significant. It is therefore conceivable that optimum results (100% recovery) will be produced at the +2 coded level for all variables while the manipulation of each parameter poses no adverse effect on the other or to the obtained recoveries. Following these observations, a reduced model was constructed taking into account only the significant parameters (**Table 3**).

Univariate Approach. Because all three studied parameters produced increasing recoveries with no interaction among each other, they were also examined individually in a one-variable-at-a-time approach in order to decide upon the optimum conditions with the aim to obtain recoveries exceeding 90%.

With regard to temperature, which in the multivariate approach produced the most positive coefficient (steepest increase), it was shown that temperatures above 60 °C produced acceptable recoveries. After all, the viscous nature of honey dictates the use of elevated temperatures, which enable easier manipulation of the sample. On the other hand, in the strong acidic environment applied for the anionic surfactant CPE, an excessive increase in temperatures produces dark-colored products due to the caramelization of sugars, which make the boundaries between the aqueous and the surfactant rich phase difficult to distinguish and the collection of the latter troublesome. In this respect, the temperature was kept at 65 °C. Similarly, the time needed to produce acceptable recoveries was univariately optimized showing again that at values exceeding 30 min, recoveries approached 100%. Therefore, 30 min was selected in order to minimize the average time of analysis as much as possible. Finally, the amount of NaCl was set at 1 g for a final volume of 50 mL as for the selected time and temperature that this NaCl concentration value produced quantitative recoveries while accelerating phase separation as well. Further addition of NaCl gave no actual improvement.

Analysis of Spiked and Real Samples. To assess the method's accuracy, a *p*-DCB free honey [*p*-DCB < 0.1 μg/kg

as determined after purge and trap GC-MS analysis according to Tananaki et al. (*7*)] was spiked with various amounts of *p*-DCB, thoroughly homogenized in a stomacher, and subsequently analyzed following the proposed protocol. **Figure 1** shows the chromatograms obtained after the analysis of the *p*-DCB free honey and the 10 and 30 μg/kg level spikes. (Inserted graphs are magnifications of the *p*-DCB peaks, showing that they are easily integratable by HPLC software support) Results at each level were produced by duplicate runs for five subsamples at each level in order to also estimate the uncertainty of each measurement. As can be seen from the results depicted in **Table 4**, the recoveries are high, exceeding 90% in the low μg/kg area, which is of legislative interest, while a slight decrease is recorded at higher concentrations. Following the recovery study, we applied the proposed methodology to the analysis of 10 honey samples obtained from different locations all over Greece. As shown in **Table 5**, only two of these samples contained amounts of *p*-DCB exceeding the legislative threshold of 10 μg/kg. **Figure 2** shows the chromatogram obtained from the analysis of sample 6. According to the information provided by the honey suppliers, both samples were obtained by producers officially using moth repellant to preserve the honeybee combs while the other eight samples originated from producers that have abandoned this method of preservation for at least 2 years.

Investigation for Gas Chromatographic Analysis' Possibility. As a tentative experimental possibility, an attempt was also made to verify the presence of *p*-DCB in the anionic surfactant micellar structures by GC−flame ionization (FI) detection. The reasoning behind this effort is that *p*-DCB is regarded as a volatile organic compound often included in commercially available GC protocols, while GC apparatus coupled with FI detectors are very common instruments in an average analytical laboratory. Following a working protocol recently introduced and applied in our laboratory (*17*, *18*), we obtained micellar extracts in isooctane readily injectable into the GC apparatus. **Figure 3** shows that the *p*-DCB peak (although at the high concentration of 2.5 mg/kg) appears clearly in both standard and sample (honey) extracts even in the presence of other dichlorobenzenes.

No optimization or further investigation was performed since this would be the content of another work, and our aim was simply to spot the possibility for GC determination of *p*-DCB after anionic surfactant CPE.

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