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On-line coupling of supercritical CO_2 extraction with reversed-phase liquid chromatography for the quantitative analysis of analytes in aqueous matrices

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Abstract

The first report of on-line coupled supercritical fluid extraction (SFE) with reversed-phase liquid chromatography for the quantitative analysis of analytes in aqueous matrices is described. Two commercial systems (e.g. SFE and HPLC) were connected via a single six-port injection valve. By using water to eliminate residual decompressed CO_2 gas in the solid-phase extraction trap, quantitative extraction and transfer were achieved for the target analytes (progesterone, phenanthrene, and pyrene) spiked in water, as well as in real samples (urine and environmental water). During each extraction, no restrictor plugging was realized. Extraction temperature and pressure were optimized. Different amounts of salt were added to the aqueous matrix to enhance ionic strength and thus extraction efficiency. Methanol and 2-propanol were used as CO_2 modifiers. Compared with dynamically mixing modifier with the CO_2 extraction fluid, pre-spiking the same amount of modifier in the extraction vessel enhanced the recovery ~30% for progesterone, phenanthrene, and pyrene due to a "co-extraction effect".
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1. Introduction

Supercritical fluid extraction (SFE) as a sample preparation technique in analytical chemistry has received a great deal of attention in the last decade [1]. Most SFE applications are focused on solid samples. SFE of aqueous matrices could give rise to a number of uses such as the isolation of industrial chemicals from waste water; pesticides and herbicides from run-off waters; and contaminants from drinking water. It might also prove useful in the analysis of other liquid matrices such as beverages, biological samples, and pharmaceutical fluids [1]. SFE of aqueous samples for analytical purposes has received less attention compared to solid samples. The major difficulty lies in the confinement of the matrix, therefore, the extraction cell must be of a geometry that retains 100% of the bulk water during the extraction [2]. Otherwise restrictors will suffer from plugging caused by ice formation during supercritical fluid expansion.

Up to now, only three methods have been reported for direct aqueous SFE. These are (a) phase separator method, (b) counter-current method, and (c) liquid–liquid extraction method. Thiebaut et al. used a novel phase separator to extract 4-chlorophenol and phenol from water with supercritical CO_2 [3]. The segmented mixture of CO_2 and water was separated in a phase separator which consisted of an upper hydrophobic membrane (usually polymer) and a lower hydrophilic surface (usually stainless steel). The liquid-fluid mixture passed along the groove in the separator and separation was achieved based on the difference in wettability of the two surfaces. However, sample recovery was less than 60%. Counter-current SFE takes advantage of the density difference of supercritical fluid and water [4]. The more dense fluid (water) can be transported in one direction, while the other fluid (supercritical fluid) can be transported in the opposite direction. The two materials meet and mix inside the extractor. Solutes that are soluble in supercritical CO_2 are brought out from the top, while water will elute out from the bottom. This technique allows larger sample volumes to be extracted. Counter-current SFE of small quantities of water

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(<100 ml) is not feasible because of the lack of suitable technology.

Up to now, the most successful design for analytical scale SFE of aqueous matrices is a liquid–liquid extraction configuration, first reported in 1989 [1,2,5–13]. Supercritical CO_2 was allowed to enter through a tube thus passing to the bottom of the vessel where it mixed with the aqueous medium to extract the compounds of interest. Due to the lower density of supercritical CO_2 compared to water, the supercritical fluid rose to the top of the extraction vessel and exited through a second tube to either a trap or re-circulation pump. This type of extractor was used to qualitatively extract nitrogenous bases [1], phenols [2], phosphonate [5], active components in drugs [6], metal chelates [7], steroids [8], polycyclic aromatic hydrocarbons (PAHs) [9], chlorinated pesticides and polychlorinated biphenyls (PCBs) [10] from an aqueous matrix.

On-line coupling of SFE and chromatographic techniques is beneficial for trace analysis, since all of the extract is transferred to the separation column and to the detector. In addition, this method is far less labor intensive than off-line analysis and the opportunity for the sample to become contaminated, volatilized, or degraded is minimized [14,15]. Recently, Pól and Wenclawiak reported on-line coupling of continuous SFE with HPLC for the analysis of an aqueous pyrethrin solution [16]. It offered extraction of unlimited sample volume, but for a limited sample (several milliliters or less), it was not suitable. In former research, we reported a novel interface for the direct coupling of SFE of a solid and HPLC [17,18]. Only one six-port injection valve was used to connect two commercial systems (SFE + HPLC). Quantitative extraction and transfer were achieved for polynuclear aromatic hydrocarbons and polymer additives spiked in a solid matrix. In this paper, we have tested the suitability of the on-line SFE–HPLC method for direct aqueous extraction for ~milliliter level sample. Two types of compounds, of pharmaceutical interest (caffeine, progesterone) and of environmental interest (phenanthrene, pyrene), were quantitatively extracted and transferred from water and urine as well as a real environmental sample. The pre-spiked matrix method was surprisingly found to be very beneficial for the direct aqueous extraction.

2. Experimental

2.1. Apparatus

An Isco-Suprex (Lincoln, NE, USA) Prepmaster supercritical fluid extraction system equipped with Accutrap and modifier pump was used for all parts of the study. SFE/SFC-grade carbon dioxide with 2000 p.s.i. helium head pressure was provided by Air Products and Chemical Inc. (Allentown, PA, USA; 1 p.s.i. = 6894.76 Pa). Extractions were performed using a 12 cm × 1.1 cm I.D. stainless steel vessel (10 ml volume, specific design can be found in ref. [1]). The vessel was filled with 5 ml water and homogeneously spiked with 10 μl stock methanol solution by shaking the vessel for ~1 min. The SFE variable restrictor was heated to 60 °C for all extractions. A 10 cm × 0.2 cm I.D. stainless steel column filled with C_{18} (Isolute Sorbent, 40–70 μm particles) was used as the SFE trap. If not noted, the trap temperature was kept at 20 °C for collection and desorption of analytes. In this study, the flow rate of liquid CO_2 prior to being heated past the critical temperature was set at 1 ml/min. During analyte desorption, the flow rate of rinse water was 1 ml/min for 4 min.

An Agilent 1050 HPLC system (Wilmington, DE, USA) with programmable multi-wavelength UV detector was used to analyze the extracts. A Phenomenex C_{18} column (Torrance, CA, USA), 250 mm × 4.6 mm with 5 μm particles was used for the separation. The mobile phase was acetonitrile–water (30:70, v/v), then to 80:20 at 10 min, to 100% acetonitrile at 20 min, kept for 2 min. The UV detection wavelength was set at 250 nm. Direct injection of standards into the LC column was accomplished by using a Valco injection valve (Houston, TX, USA) with a 10 μl sample loop.

Experimental details concerning the hyphenated SFE–HPLC interface appear elsewhere [17,18].

2.2. Chemicals and real sample

HPLC-grade methanol, acetonitrile, and water (Burdick & Jackson, Muskegon, MI, USA) were used. A mixture of caffeine, progesterone, phenanthrene, and pyrene (Aldrich, Milwaukee, WI, USA) in methanol was prepared as a stock solution, with concentrations of 800, 80, 10, 60 μg/ml, respectively.

Urine and environmental water were chosen as two real matrices. Urine was provided by a healthy donor. During the experiment, 1 ml urine, 2.2 ml HPLC-grade water and 1.8 ml methanol were mixed in the vessel with 10 μl of 20 μg/ml progesterone stock solution (the absolute concentration in the vessel was 40 ng/ml). The environmental water sample was collected at the Virginia Tech Duck Pond nearby. Environmental water (1.0 ml), 2.2 ml HPLC water, and 1.8 ml methanol were mixed in the vessel with 10 μl of 2 μg/ml phenanthrene and 12 μg/ml pyrene stock solution (the absolute concentrations in the vessel were 4 and 24 ng/ml for phenanthrene and pyrene, respectively). The extraction was carried out by using 15 g CO_2 as the extraction media.

3. Results and discussion

3.1. Optimization of extraction recovery

3.1.1. Pure supercritical CO_2 as the extraction media

On-line SFE–HPLC of the four analytes spiked into water (i.e. extracted with CO_2, collected on the trap, mobilized from the trap by the mobile phase, and analyzed via LC)

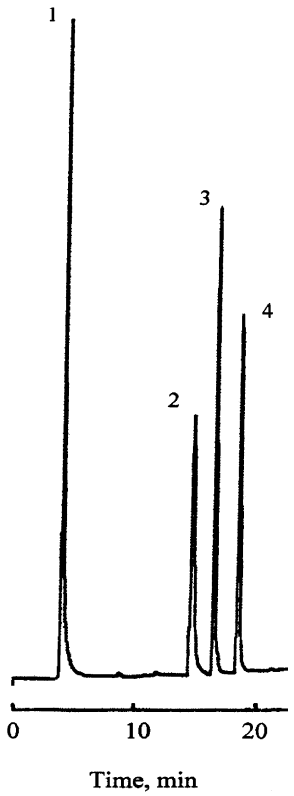

Fig. 1. Chromatogram of four spiked compounds via on-line SFE–HPLC. (1) Caffeine, (2) progesterone, (3) phenanthrene, and (4) pyrene. C_{18} column (250 mm × 4.6 mm, 5 μm) was used for separation. The mobile phase was acetonitrile–water (30:70, v/v), then to 80:20 at 10 min, to 100% acetonitrile at 20 min, kept for 2 min. Flow rate was 1.0 ml/min. The UV detection wavelength was set at 250 nm. SFE: 350 atm CO_2 at 60 °C, 30 g CO_2 consumed for dynamic extraction. C_{18} was used as sorbent in the trap. Trap temperature for collection and for desorption both were 20 °C. Four milliliters water was delivered to replace residual CO_2 in the trap. Ten microliters stock solution was spiked into water.

yielded the trace shown in Fig. 1. In this design, the extraction mode is similar to liquid–liquid extraction where the upper phase is supercritical fluid and the lower phase is liquid. The extraction process is believed to be partitioning of the analytes between the two phases and the analyte's partition coefficient is the decisive parameter. The coefficient can be roughly estimated from the ratio of the analyte's solubility in these two phases. Among the four target compounds, caffeine is the most polar one and also has the highest water solubility due to its strong hydrogen bonding with water.

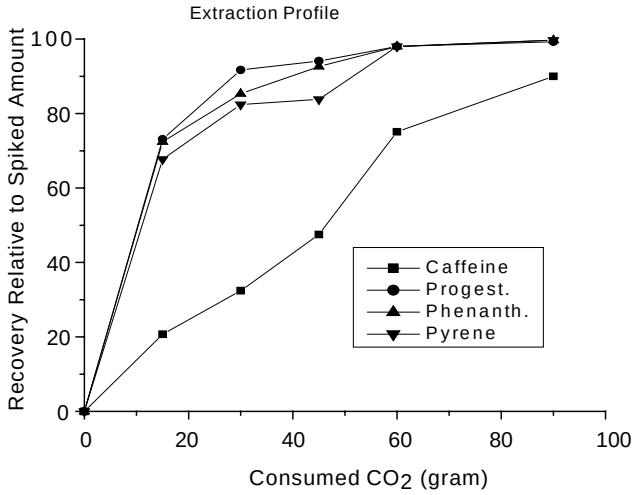

Fig. 2. Extraction profiles (see Fig. 1 for HPLC and SFE conditions).

The extraction profile shown in Fig. 2 reveals that progesterone, phenanthrene, and pyrene can be exhaustively extracted with 60 g of CO_2; however, for caffeine, the recovery was still only 90% when 90 g of CO_2 was consumed.

An optimum extraction temperature was explored. As the temperature rises, the analytes become more soluble in water; at the same time, the volatility of those compounds also will increase, which facilitates mass transfer to the upper phase. The density and solvating power of supercritical carbon dioxide also have an effect. They will decrease when temperature increases. The extraction recoveries at three different temperatures are shown in Table 1. The intermediate temperature (60 °C) was found to be more efficient than either 40 °C or 80 °C.

The influence of extraction pressure was also investigated by changing the pressure from 250 to 450 atm at 100 atm intervals with an extraction temperature of 60 °C (Table 2; 1 atm = 101 325 Pa). The highest recovery was achieved at 350 atm (e.g. the intermediate pressure). This result may be rationalized in terms of the cohesive energy of supercritical fluid. When pressure increases, the cohesive energy of the supercritical fluid also increases. In the aqueous matrix, the greater cohesive energy translates into less interaction (or mixing) of the fluid with the matrix because of decreased fluid surface area. Smaller surface area reduces the chance of supercritical fluid to contact the

Table 1
Percent recovery vs. extraction temperature (10 min dynamic extraction, other conditions were the same as described in Fig. 1)

	350 atm, 40 °C, 0.94 g/ml		350 atm, 60 °C, 0.87 g/ml		350 atm, 80 °C, 0.79 g/ml	
	Recovery (%)	R.S.D.[a] (%)	Recovery (%)	R.S.D.[a] (%)	Recovery (%)	R.S.D.[a] (%)
Caffeine	9.2	7.9	9.8	8.4	7.3	8.6
Progesterone	41.1	4.3	50.6	4.2	32.1	5.4
Phenanthrene	45.3	5.0	56.7	5.3	33.5	6.0
Pyrene	39.2	5.2	48.8	5.7	29.3	5.6

[a] $n = 3$.

Table 2
Percent recovery versus extraction pressure (10 min dynamic extraction, other conditions were the same as described in Fig. 1)

	250 atm, 60 °C, 0.79 g/ml		350 atm, 60 °C, 0.87 g/ml		450 atm, 60 °C, 0.92 g/ml	
	Recovery (%)	R.S.D.[a] (%)	Recovery (%)	R.S.D.[a] (%)	Recovery (%)	R.S.D.[a] (%)
Caffeine	8.0	8.9	9.8	8.4	7.9	8.0
Progesterone	42.4	5.1	50.6	4.2	42.6	4.9
Phenanthrene	47.5	5.2	56.7	5.3	47.9	5.5
Pyrene	39.9	5.6	48.8	5.7	39.3	5.5

[a] $n = 3$.

analytes, so lower efficiency was observed at 450 atm. When the pressure increased from 250 to 350 atm, the density of supercritical fluid increased with higher solvating power, and in this pressure range, surface area is not a major factor, so we observed higher extraction efficiency at 350 atm than at 250 atm due to the increased CO_2 density.

"Salting out" is widely used in liquid–liquid extraction [19]. We explored the extraction of our target analytes at 1 and 3% (w/w) salt (NaCl) concentration. However, there were no obvious changes in extraction efficiency and extraction kinetics. A similar result was observed when PCBs, organochlorine pesticides (OCPs), and pyrethrines were extracted from water (2% NaCl) [16]. The probable reason maybe because at low salt concentration, the effect of "salting out" is not significant enough to affect the recovery of the analytes. At higher salt concentration, we may observe the enhancement in the extraction efficiency, but with an SFE instrument, the operation at high salt concentration is adventurous. Salt crystals may deposit and plug the restrictor and tubing, when supercritical fluid is decompressed causing its solvating power to be decreased.

The flow rate of supercritical fluid has an influence on trap efficiency. In our study, the mass of supercritical CO_2 was kept at 15 g. Under these conditions, recoveries decreased slightly when the measured CO_2 liquid flow rate at the pump was increased from 1 to 3 ml/min (Fig. 3). The trap apparently lost some efficiency at the high flow rate.

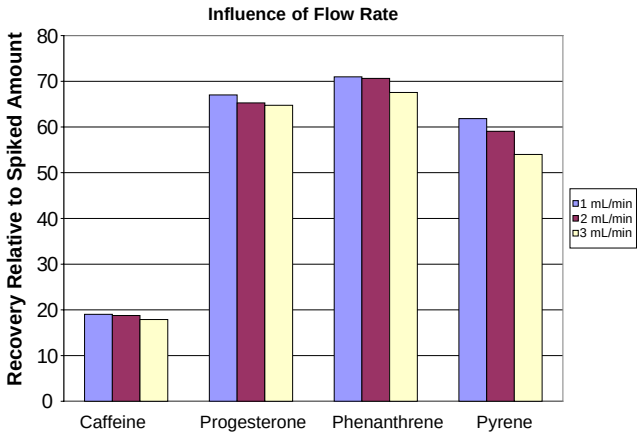

Fig. 3. The influence of supercritical fluid flow rate on recovery (15 g CO_2 consumed for dynamic extraction, other conditions were the same as described in Fig. 1).

3.1.2. *Modified supercritical CO_2 as the extraction media*

Organic solvent modified supercritical CO_2 is often used for the extraction of polar analytes. Modifier can be dynamically added to the fluid by an additional pump, or it can be pre-spiked into the vessel. For modified SFE experiments, the trap temperature was set at 60 °C to avoid the loss of trapping efficiency.

(a) *Dynamic mixing mode (via modifier pump).* Firstly, different percentages of methanol were dynamically mixed with supercritical CO_2 via a modifier pump, to test their influence on the extraction. When 15 g of CO_2 was consumed for dynamic extraction, there was no statistical difference among the data. There was a similar situation when 30 g of CO_2 was consumed. But when more CO_2 (45 g) was used (Fig. 4), the modified supercritical fluid gave slightly higher recoveries. In the dynamic modifier mixing mode, there appears to be no obvious enhancement in extraction efficiency when relatively small amounts of CO_2 (or short extraction time) were used. When more CO_2 was employed (or longer extraction time), the modifier begins to show an effect. Such phenomena probably result from the fact that at any given short time period, only a tiny amount of modifier was brought into and mixed with the aqueous matrix. Furthermore, some of dissolved modifier may have been extracted out of the aqueous matrix by supercritical CO_2. When the extraction time is longer, more modifier enters the matrix and the enhancement due to modifier begins to be visible.

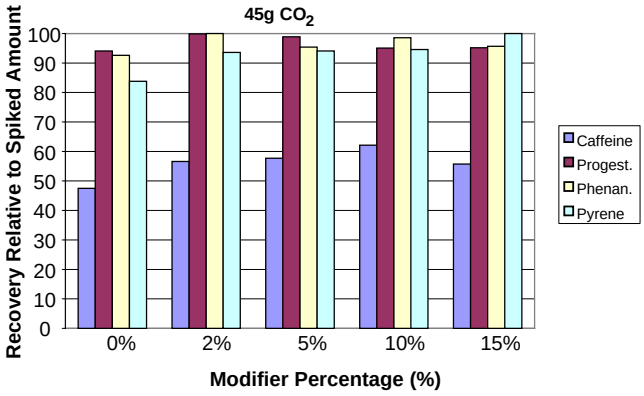

Fig. 4. The influence of modifier (methanol) percentage on recovery, 45 g CO_2 consumed for dynamic extraction (see Fig. 1 for HPLC and SFE conditions, except for the trap temperature for collection was 60 °C).

Table 3
Comparison of dynamic mixing modifier mode and pre-spiked modifier mode on percent recovery

	0.3 ml Methanol spiked in vessel		2% Methanol dynamic mixing (consumed 0.35 ml)		0.9 ml Methanol spiked in vessel		5% Methanol dynamic mixing (consumed ~ 0.9 ml)		1.8 ml Methanol spiked in vessel		10% Methanol dynamic mixing (consumed ~ 1.8 ml)	
	Recovery (%)	R.S.D.[a] (%)	Recovery (%)	R.S.D.[a] (%)	Recovery (%)	R.S.D.[a] (%)	Recovery (%)	R.S.D.[a] (%)	Recovery (%)	R.S.D.[a] (%)	Recovery (%)	R.S.D.[a] (%)
Caffeine	26.5	7.1	17.2	5.5	27.6	2.4	20.4	4.4	28.7	4.6	21.2	1.2
Progesterone	92.9	2.3	71.3	5.3	99.3	0.7	72.9	0.4	100.1	1.0	69.9	1.8
Phenanthrene	82.9	1.1	71.1	7.2	91.4	3.8	69.6	6.2	100.0	0.4	73.8	3.6
Pyrene	75.9	6.4	69.9	7.7	90.8	6.1	65.3	6.3	100.1	1.0	64.4	5.2

See Fig. 1 for HPLC and SFE conditions, except the trap temperature for collection was 60 °C. Methanol was used as modifier.
[a] $n = 3$.

(b) *Pre-spiking mode (via spiking modifier in the vessel).* Pól and Wenclawiak reported that in the continuous SFE mode, the addition of 2 and 5% methanol slightly increased extraction recovery; the addition of 10% methanol decreased the recovery [16]. The pre-spiked matrix modifier mode was then investigated in our design. To equally compare with the dynamic mixing mode (for 15 g CO_2 with 2, 5 and 10% modifier, the total methanol consumed was 0.35, 0.9 and 1.8 ml, respectively), the same amount of methanol was pre-spiked into the vessel that contained water filled to a total volume of 5 ml (15 g CO_2 was the extraction media). When the extraction was preformed, surprisingly the pre-spiked matrix method achieved 10–30% enhancement in extraction efficiency (except for caffeine where the enhancement was less than 10% increase), When 1.8 ml of methanol was pre-spiked, an exhaustive quantitative extraction was achieved for progesterone, phenanthrene, and pyrene (Table 3). Their recovery was enhanced as high as 30–35%, compared to the data obtained with 10% modifier in the dynamic mixing mode. This distinct improvement is probably due to a "co-extraction effect". At the beginning of the extraction, methanol has a relatively high concentration in the vessel and is homogeneously mixed with water and analytes. Due to the high solubility of methanol in supercritical CO_2, it maybe quickly extracted from the water matrix, and at the same time the analytes maybe co-extracted along with the methanol. For caffeine, which has a strong interaction (hydrogen bonding) with water, only a slight increase in recovery was observed. 2-Propanol was also tested as a modifier and it achieved a similar result to methanol.

3.2. Linearity

The linear range of C_{18} trap capacity (10 cm × 0.2 cm I.D.) was also investigated at optimized conditions (Table 4). The linear range was $\sim 10^2$ for caffeine and pyrene, and $\sim 10^3$ for progesterone and phenanthrene. All the correlation coefficients were larger than 0.99.

3.3. SFE–LC method for the real samples

Finally to evaluate this hyphenated system for more complicated matrices, the target analytes in real samples were quantitatively analyzed. Based on the matrix-spiked results, 1.8 ml of methanol as modifier was pre-spiked into the vessel containing the real matrix. Each time 1 ml of the real liquid matrix and 2.2 ml HPLC-grade water were added to the vessel (total volume is 5 ml). Ten microliters of the stock analyte solution was then added and well mixed. Figs. 5a and 6a show the HPLC chromatograms of blank urine and the environmental water sample, which were extracted via on-line SFE–LC. Figs. 5b and 6b show the chromatographic peaks for 40 ng/ml progesterone in urine as well as 4 ng/ml

Table 4
Linear range, correlation coefficient and precision data

Compound	Linear range (ng)[a]	Correlation coefficient	R.S.D. (%, $n = 3$)[b]	LOD (ng)[c]
Caffeine	3000–300000	0.9989	10.5	50
Progesterone	16–16000	0.9998	4.5	1
Phenanthrene	2–2000	0.9997	5.4	0.1
Pyrene	2–1000	0.9996	5.7	0.5

[a] Amount of analytes spiked in water.
[b] Average of R.S.D.s at each measured concentration.
[c] Limit of detection.

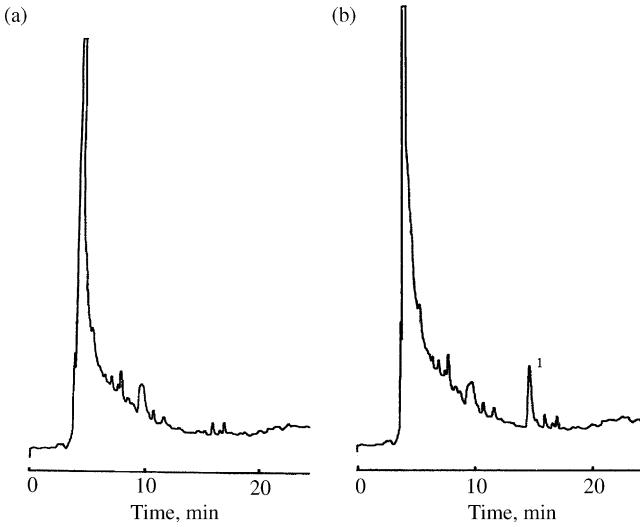

Fig. 5. Analysis of progesterone in urine sample via on-line SFE–HPLC. One milliliter urine, 2.2 ml HPLC-grade water and 1.8 ml methanol were well mixed in the vessel with 10 μl of 20 μg/ml progesterone stock solution (the absolute concentration in vessel is 40 ng/ml). Fifteen grams CO_2 consumed for dynamic extraction. The trap temperature for collection was 60 °C. Other conditions were same with Fig. 1. (a) Blank urine sample; (b) sample spiked with progesterone. 1 = Progesterone.

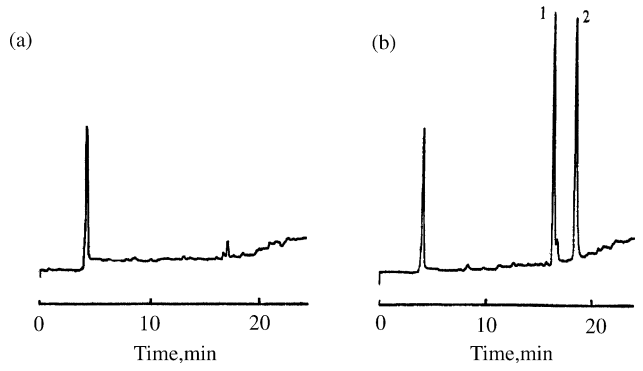

Fig. 6. Analysis of phenanthrene and pyrene in environmental water via on-line SFE–HPLC. One milliliter environmental water, 2.2 ml HPLC-grade water and 1.8 ml methanol were well mixed in the vessel 10 μl of 2 μg/ml phenanthrene and 12 μg/ml pyrene stock solution (the absolute concentration in vessel is 4 and 24 ng/ml for phenanthrene and pyrene, respectively). Other conditions were same with Fig. 5. (a) Blank environmental water; (b) sample spiked with phenanthrene and pyrene. 1 = Phenanthrene; and 2 = pyrene.

Table 5
Recovery and precision data for the analysis of real sample

	Urine sample, progesterone	Environmental water	
		Phenanthrene	Pyrene
Recovery (%)	100.1	100.0	99.8
R.S.D. (%, $n = 3$)	4.1	1.0	2.2

Conditions as in Figs. 5 and 6.

phenanthrene and 24 ng/ml pyrene in the environmental water. Quantitative extraction was achieved in less than 20 min for each analyte, with reasonable experimental error (Table 5).

4. Conclusion

In this study, on-line SFE–HPLC for the extraction and quantitative analysis of analytes in small volumes of aqueous sample was performed. Experimental parameters were evaluated and optimized. Pre-spiking modifier into the vessel followed by extraction with pure SF CO_2 was found to be very efficient to enhance the extraction recovery due to co-extraction effect. Quantitative extraction and transfer were achieved for the target analytes (progesterone, phenanthrene and pyrene) spiked in water as well as in real samples. Extension of this method to additional liquid matrices such as broths, wine, and infusions is anticipated.

Acknowledgements

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