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Aqueous two-phase system based on hexafluoroisopropanol and acetonitrile for homogeneous liquid-liquid microextraction of cationic dyes

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This work demonstrates that an aqueous two-phase system (ATPS) is formed when a small amount of acetonitrile (ACN) is added to the aqueous hexafluoroisopropanol (HFIP) solution. The effect of ACN amount on the volume of the formed HFIP/ACN phase was investigated. It was also shown that relatively hydrophilic methylene blue was completely extracted into the HFIP/ACN phase whereas its extractability with conventional solvents (hexane, CH_2Cl_2 and $CHCl_3$) was significantly lower. The obtained results suggest that the HFIP/ACN phase exhibits a relatively high polarity and should be a good choice for the extraction of moderately or even highly polar compounds from aqueous samples. Finally, the developed ATPS was applied for the homogeneous liquid-liquid microextraction of four cationic dyes from river water samples prior to HPLC analysis. Under optimised extraction conditions, the enrichment factors were around 150. Calibration curves were linear ($R^2 \geq 0.9959$) for the concentration level between 0.2–0.5 and 50.0 μg/L and the detection limits were in the range 0.05–0.18 μg/L. The recoveries of the dyes for the spiked water samples were 88.6–98.5%, with the relative standard deviation values less than 9.6%.

Keywords: aqueous two-phase system, hexafluoroisopropanol, acetonitrile, microextraction, cationic dyes

INTRODUCTION

Liquid-liquid extraction (LLE) is one of the oldest and still among the most popular techniques in the preparation of samples for analysis [1]. In the conventional LLE, hydrophobic sample constituents are extracted from aqueous samples with a water-immiscible organic solvent, such as hexane, diethyl ether, ethyl acetate, chloroform and some others. However, the limited polarity range of these solvents restricts their use for the extraction of more hydrophilic compounds. Over the last two decades, several new classes of solvents, such as ionic liquids [2], deep eutectic solvents [3], and aqueous two-phase systems (ATPS) [4], have been designed and introduced in LLE and its miniaturised techniques as alternatives to traditional organic solvents. Among the new generation extractants, ATPSs are perhaps the most promising ones due to their wide polarity range, tunable physicochemical properties and simplicity of preparation [5].

ATPSs are systems where two immiscible liquid phases are formed by mixing at least two water-soluble compounds such as polymer–polymer, polymer–salt, ionic liquid–salt, alcohol–salt, acetonitrile–carbohydrate, and some others [4]. Since the 1980s, ATPSs have been widely used for

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the preparative separation and purification of proteins, enzymes, DNA, viruses and other biomolecules [5, 6]. In recent years, these systems have received extensive attention in the field of analytical chemistry. Both, conventional ATPS-based LLE [4, 7] and its miniaturized version, called homogeneous liquid-liquid microextraction (HLLME) [8], have been successfully applied for the preconcentration of various compounds from aqueous samples. Although these alternative systems are generally simple, fast, inexpensive, and have potential for the extraction of compounds covering a wide polarity range, they also present certain limitations. Most of the ATPS-based solvents proposed so far are non-volatile, lighter than water, highly viscous liquids and their use for conventional LLE and especially for HLLME techniques is limited. Due to their low volatility, they are incompatible with the gas chromatography technique. Solvents lighter than water remain at the top of the sample solution after the phase separation, thus forming a thin film that is difficult to collect. Finally, their high viscosity hinders the mass transfer between phases resulting in a lower extraction efficiency and makes it difficult to handle the extracts prior to analysis. Thus, the search for new ATPSs with valuable physicochemical properties is of great interest.

In this study, a novel hexafluoroisopropanol (HFIP) based ATPS was developed using acetonitrile (ACN) as the phase separation inducing agent. To the best of our knowledge, this is the first ATPS consisting of two hydrophilic organic solvents. The developed ATPS was applied for the HLLME of four cationic dyes from water samples prior to the analysis by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Reagents and solutions

Hexafluoroisopropanol (purity ≥99%) and acetonitrile (LC-MS grade) were purchased from Merck Life Science (Merck KGaA, Darmstadt, Germany). Crystal violet, malachite green, methylene blue, rhodamine B and triphenyltetrazolium chlorides were acquired from Sigma-Aldrich (St. Lous, MO, USA) with a purity higher than 95%. Ultrapure water was obtained from a Milli-Q water purification system from Millipore (Bedford, MA, USA).

Stock solutions (200 mg/L) of all the analytes were prepared in methanol/H_2O (1:1, v/v) and stored in a refrigerator at 4°C. Working solutions were prepared by diluting the stock solutions in Milli-Q water before use.

Instrumentation

Chromatographic separations were performed on an Agilent 1290 Infinity II LC system (Agilent, Waldbronn, Germany) equipped with a ternary pump, thermostatted column compartment, photodiode array detector and autosampler. InfinityLab Poroshell 120 EC-C18 (3.0 × 150 mm, 2.7 μm, Agilent) column maintained at 25°C was used in the experiments. The separations were performed at a flow rate of 0.5 mL/min. Cationic dyes were separated with an ACN/water mobile phase containing 0.1% (*v/v*) formic acid under linear gradient elution conditions. The composition of the mobile phase was changed from 30% ACN to 100% ACN in 7 min. The injection volume was 10 μL. Data acquisition was performed by the Agilent OpenLAB CDS software.

Procedures

Water samples were collected in September 2023 from the Neris River in two different locations within the area of Vilnius. Both samples were analysed on the same day after sampling. Before microextraction, the samples were filtered through a 0.7 μm glass fibre filtre. All measurements were performed in triplicate and the mean values were reported.

ATPS formation experiments were performed at room temperature. 5.0 mL of water was placed into a 10 mL glass centrifuge tube and specified amounts of HFIP and ACN were sequentially added. The phases were separated by centrifugation at 3000 rpm for 5 min and the volume of HFIP/ACN phase was measured using a microsyringe.

For the extraction efficiency measurements, 5.0–7.5 mL of the aqueous solution of analytes with concentrations of 2.0 mg/L was placed into a 10 mL glass centrifuge tube, and 0.3–0.4 mL of HFIP and 0.3–0.4 mL of ACN were sequentially added. The mixture was shaken manually for 30 s, resulting in the formation of an emulsion. The phases were separated by centrifugation and

the upper aqueous phase was then analysed using the HPLC technique.

The extraction efficiency (EE) of each analyte was calculated according to the following equation,

$$EE(\%)=\frac{c_0.V_0 - c_i.V_i}{c_0.V_0}\cdot 100\%,$$

where c_0 and c_i are the concentration of the analyte in the aqueous phase before and after extraction, respectively. V_0 and V_i represent the volume of the aqueous phase before and after the extraction, respectively. The final concentrations of the analytes in the aqueous phase were measured using the HPLC technique.

The final microextraction procedure was carried out as follows: 7.5 mL of the water sample was placed into a 10 mL glass centrifuge tube and spiked with the internal standard (triphenyltetrazolium chloride) at 10 µg/L. Then, 0.4 mL of HFIP and 0.4 mL of ACN were sequentially added and the resulting mixture was shaken manually for 5 s. The phases were separated by centrifugation and the upper aqueous phase was removed with a syringe. Finally, the extract was evaporated to dryness under a stream of nitrogen, the residue was dissolved in 50 µL of the initial mobile phase and analysed using the HPLC technique.

RESULTS AND DISCUSSION

A schematic illustration of the proposed ATPS formation procedure is shown in Fig. 1. A heavier than water HFIP-rich liquid phase is formed when a specific amount of the inducing agent (ACN) is added to the aqueous HFIP solution. Although the exact mechanism for this phenomenon is unknown, it is believed that the main driving force of phase separation is a competitive hydrogen bonding between water, HFIP and ACN. HFIP is a strong hydrogen bond donor (even stronger than water), but a weak hydrogen bond acceptor [9]. Acetonitrile, in contrast, exhibits a strong hydrogen bond acceptor ability. The hydrogen bonding interaction between HFIP and ACN is stronger than that between HFIP and water. Thus, ACN molecules displace water molecules from the hydration layer of HFIP. In this case, the HFIP molecules likely cluster and even form micelle-like assemblies with the fluorine groups aggregating toward the centre of the cluster while oriented at the surface hydroxy groups are solvated by ACN. Such clusters of HFIP molecules provide a hydrophobic local environment. This results in the formation of the immiscible with water HFIP-rich phase.

In the liquid-liquid microextraction techniques, target analytes are usually extracted from 5–10 mL of water samples using small volumes (~50–200 µL) of extraction solvents [10]. In the ATPS-based microextraction system, the extraction solvent is formed *in situ*. Therefore, it is important to know what amount of the ATPS inducing agent generates the required extractant volume. For this reason, the effect of ACN amount on the volume of the formed HFIP/ACN phase was investigated. As shown in Fig. 2, the HFIP phase volume increases gradually as the volume of ACN increases from 0.1 to 0.7 mL and then remains almost unchanged at higher than 0.7 mL volumes.

As already mentioned in the Introduction, conventional water-immiscible solvents, such as

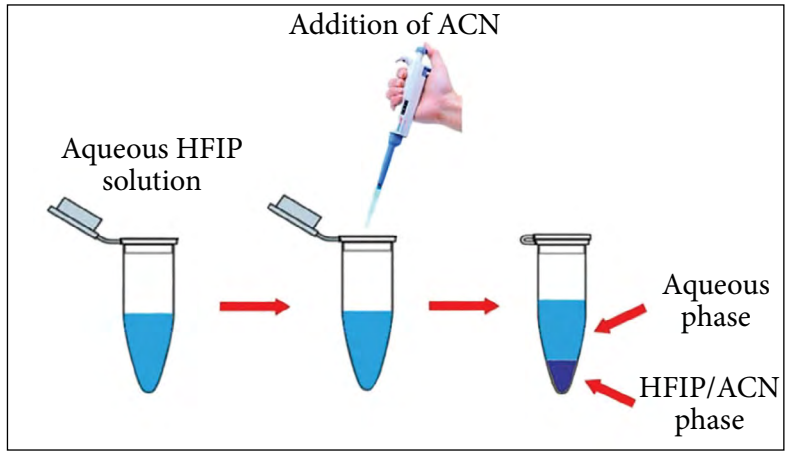

Fig. 1. Schematic presentation of an aqueous two-phase system formation procedure

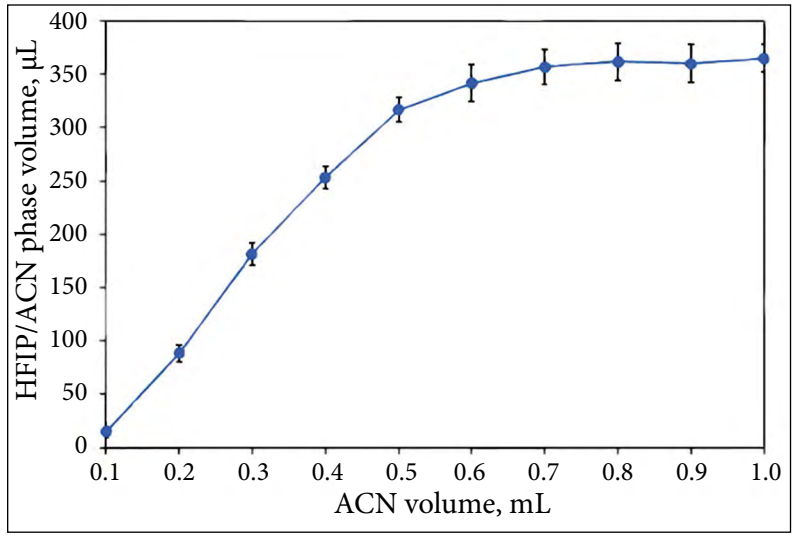

Fig. 2. Effect of ACN volume on the formed HFIP/ACN phase volume. Aqueous phase volume 5.0 mL; HFIP volume 0.3 mL

hexane, diethyl ether, chloroform and some others, exhibit a limited polarity range and this restricts their use for the extraction of more hydrophilic compounds. To assess the relative polarity of the proposed ATPS it was tested for the extraction of moderately hydrophilic (log $P = 0.75$) cationic methylene blue from an aqueous solution. Three conventional solvents (hexane, CH_2Cl_2 and $CHCl_3$) were also employed under the same extraction conditions. For each solvent we have selected such initial volume that after extraction gave the final extract volume of 180±10 µL. Even visual inspection (Fig. 3) shows that the cationic methylene blue seems to be completely extracted into the HFIP/ACN phase whereas its extractability with conventional solvents was significantly lower. The obtained extraction efficiencies (EE) are compared in Table 1. It was not possible to evaluate higher than 98% EE data because in this case the concentration of dye in the aqueous phase after extraction was below the limit of quantification. It can be observed that for three extractants the obtained EE values showed a good correlation with their log *P* values: more polar solvents demonstrated a better extractability. Thus, although the log P value for the HFIP/ACN phase is unknown, the obtained results suggest that it exhibits a relatively high polarity and, consequently, should be a good choice for the extraction of moderately or even highly polar compounds from aqueous samples.

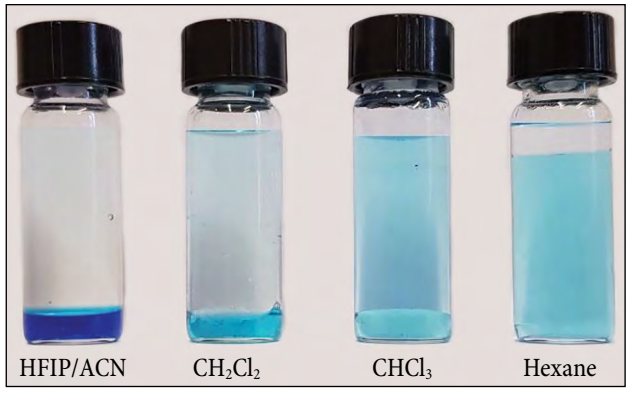

Fig. 3. Partition behaviour of the methylene blue in different extractants. Hexane is the top phase

Table 1. **Extraction efficiencies of methylene blue (2.0 mg/L) from aqueous solution with different extractants. Aqueous sample volume 5.0 mL; extract volume 180±10 µL; extraction time 30 s**

Extractant	log P [11]	EE, %
HFIP/ACN	–	≥98
CH_2Cl_2	1.25	46.4
$CHCl_3$	1.97	15.2
Hexane	3.90	0

Synthetic dyes are widely used in many industries including food, cosmetics, textiles, pharmaceuticals, and leather tanning [12]. The effluents containing synthetic dyes were reported to cause a series of adverse effects on aquatic environment and human health [13]. Therefore, it is essential to develop relatively fast and sensitive methods for the trace analysis of synthetic dyes in waste and environmental waters. The developed ATPS was

employed for the HLLME of four synthetic cationic dyes from real water samples combining HLLME with HPLC analysis. Triphenyltetrazolium was used as an internal standard (IS). The chemical structures of the studied compounds are shown in Fig. 4.

The extraction efficiencies of the analytes were measured under the following initial microextraction conditions: 2.0 mg/L analyte concentration, 7.5 mL aqueous sample volume, 0.4 mL HFIP volume, 0.4 mL ACN volume and 30 s extraction time. At these conditions, approximately 150 μL of the HFIP/ACN phase was formed. Higher than 98% extraction efficiencies were obtained for all five compounds.

Next, the effect of extraction time on the extraction efficiency was studied over a range of 1–30 s. No significant decrease (Table 2) in the extraction efficiency of all compounds was observed in the time range from 5 to 30 s indicating that the ATPS-based microextraction is very fast. Based on these results, 5 s of extraction time was selected for the final experiments.

Table 2. **Effect of extraction time on the extraction efficiency of dyes and internal standard (2.0 mg/L). Aqueous sample volume 7.5 mL; HFIP volume 0.4 mL; ACN volume 0.4 mL**

Extraction time, s	EE, %				
	MB	CV	IS	MG	RB
30	≥98	≥98	≥98	≥98	≥98
20	≥98	95.4	≥98	≥98	≥98
10	≥98	97.8	≥98	≥98	≥98
5	≥98	97.1	≥98	≥98	≥98
1	94.9	88.6	≥98	93.7	92.6

The analytical performance of the method was investigated under the optimised microextraction and HPLC conditions (see the Experimental Section). Following the analysis of the UV/Vis spectra of the individual analyte standards, three wavelengths (240, 560 and 595 nm) were chosen for detection. The results are summarised in Table 3. The calibration curves were linear for a concentration level between 0.2–0.5 and 50.0 μg/L with the correlation coefficients (R^2) ranging from 0.9959 to 0.9987.

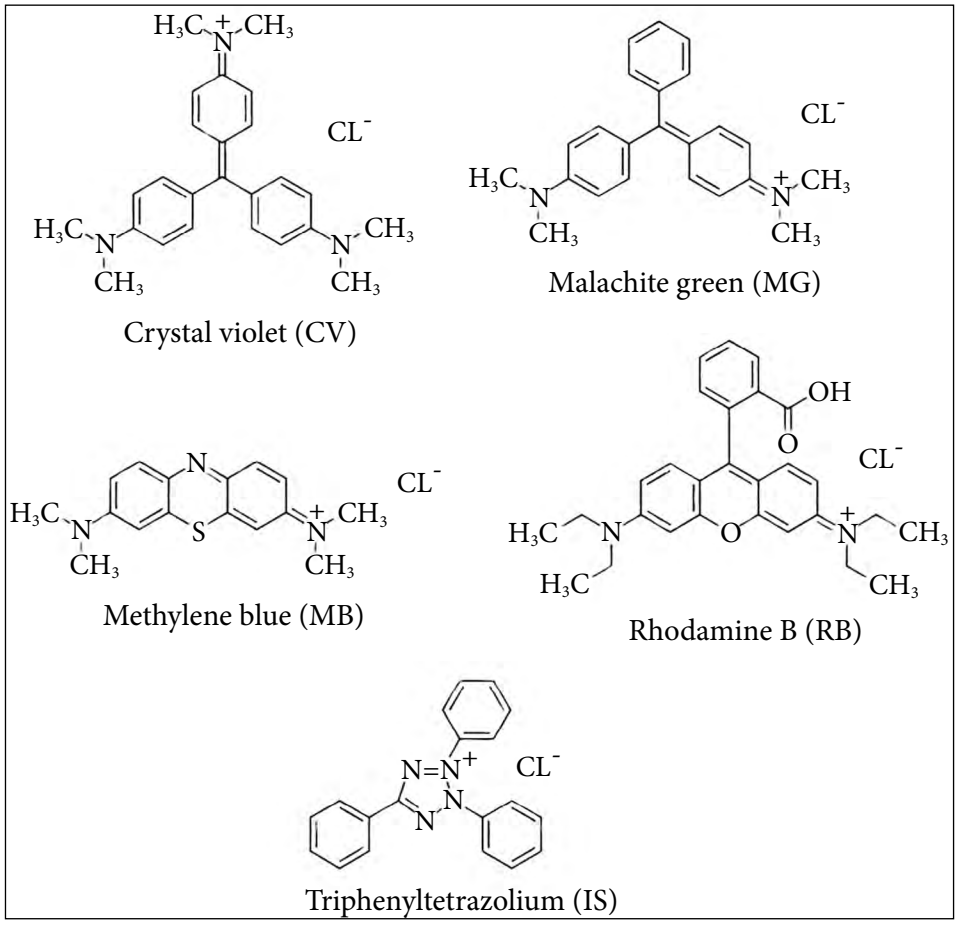

Fig. 4. Chemical structures of the studied dyes and the internal standard

The limits of detection (LODs) and the limits of quantification (LOQs), calculated based on signal to noise ratios of 3 and 10, respectively, were in the ranges 0.05–0.18 and 0.15–0.60 μg/L. The enrichment factors for all studied dyes were around 150.

Table 3. **Analytical performance characteristics of the developed HLLME-HPLC method ($n = 3$)**

Analyte	Linear range, μg/L	R^2	LOD, μg/L	LOQ, μg/L
MB	0.5–50.0	0.9959	0.60	0.18
CV	0.5–50.0	0.9980	0.50	0.15
MG	0.5–50.0	0.9972	0.50	0.15
RB	0.2–50.0	0.9987	0.15	0.05

Finally, the developed method was applied for the determination of dyes in two river water samples collected from the Neris River in two different locations within the area of Vilnius. No dyes were detected in both water samples. Recovery studies were performed by spiking samples with known concentrations of dyes. The results are summarised in Table 4. The average recoveries of spiked samples were in the range from 88.6 to 98.5% with the satisfactory precision (RSD lower than 9.6%). The chromatogram of the spiked river water sample is shown in Fig. 5. The obtained results indicate

Table 4. **Recoveries of dyes from spiked Neris River water samples ($n = 3$)**

Sample	Analyte	Added, μg/L	Recovery, %	RSD, %
River water 1	MB	2.0	89.7	9.6
	CV	2.0	92.8	8.5
	MG	2.0	97.3	9.2
	RB	2.0	94.5	7.7
	MB	10.0	93.5	8.4
	CV	10.0	95.8	7.8
	MG	10.0	96.6	8.3
	RB	10.0	97.5	7.0
River water 2	MB	2.0	90.4	8.8
	CV	2.0	88.6	9.3
	MG	2.0	92.1	7.9
	RB	2.0	96.9	7.6
	MB	10.0	94.4	7.9
	CV	10.0	98.0	6.7
	MG	10.0	94.2	7.1
	RB	10.0	98.5	6.4

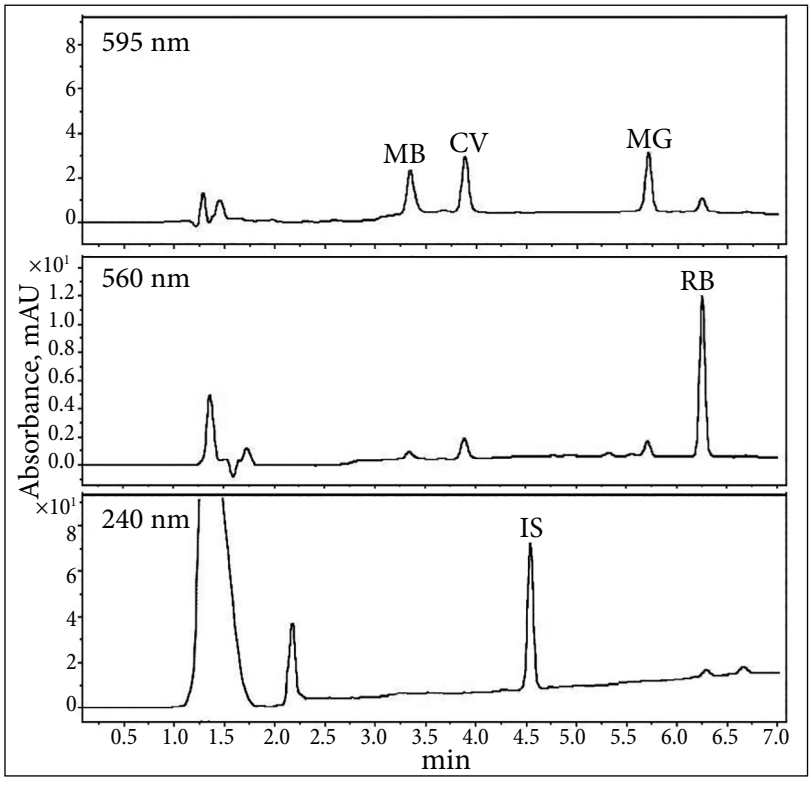

Fig. 5. Chromatogram of the spiked with 2.0 μg/L of dyes and 10.0 μg/L of internal standard river water sample. For chromatographic conditions see the Instrumentation Section

that the developed method is a promising alternative for the rapid enrichment and determination of common dyes in aqueous samples.

CONCLUSIONS

For the first time, ATPS was developed by combining two hydrophilic solvents. Compared to the conventional ATPSs based on polymers, surfactants, ionic liquids, and deep eutectic solvents, the proposed system has several advantages. The water-immiscible HFIP/ACN phase exhibits a high volatility, a higher density than water and a low viscosity. All the properties make the ATPS very promising as an extractant for conventional liquid-liquid extraction and especially for liquid-liquid microextraction techniques. Because of their high volatility, the obtained extracts are compatible with the gas chromatography technique. Moreover, the extracts can be easily evaporated and redissolved in an appropriate solvent for subsequent analysis, thus enhancing the number of suitable analytical techniques. Heavier than water extractants are preferred in liquid-liquid microextraction techniques because they sediment at the bottom of the centrifuge tube and can be simply collected by using a microsyringe. A low viscosity provides a higher mass transfer rate resulting in improved extraction efficiency. Finally, compared to the conventional water-immiscible solvents the HFIP/ACN phase possesses a higher polarity and could be used for the microextraction of compounds of a wider polarity range.

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VANDENINĖ DVIFAZĖ SISTEMA HEKSAFLUORIZOPROPANOLIO IR ACETONITRILO PAGRINDU KATIJONINIŲ DAŽIKLIŲ HOMOGENINEI SKYSČIŲ-SKYSČIŲ MIKROEKSTRAKCIJAI

S a n t r a u k a

Šiame darbe parodyta, kad į vandeninį heksafluorizopropanolio (HFIP) tirpalą pridėjus nedidelį kiekį acetonitrilo (ACN), susidaro vandeninė dvifazė sistema. Ištirta ACN kiekio įtaka susidarančios HFIP/ACN fazės tūriui. Taip pat nustatyta, kad HFIP/ACN fazė praktiškai visiškai išekstrahuoja gana hidrofilinį metileno mėlynąjį iš vandeninių tirpalų. O šio junginio ekstrakcijos efektyvumas tradiciniais tirpikliais (heksanu, CH_2Cl_2 ir $CHCl_3$) buvo žymiai blogesnis. Gauti rezultatai rodo, kad HFIP/ACN fazė pasižymi santykinai dideliu poliškumu ir turėtų būti efektyviu ekstrahentu vidutinio poliškumo ar netgi labai polinių junginių ekstrakcijai iš vandeninių mėginių.

Nauja vandeninė dvifazė sistema buvo pritaikyta keturių katijoninių dažiklių homogeninei skysčių-skysčių mikroekstrakcijai iš upės vandens ir jų nustatymui efektyviosios skysčių chromatografijos metodu. Optimizuotomis sąlygomis dažiklių sukoncentravimo laipsniai siekė apie 150. Kalibravimo kreivės buvo tiesinės 0,2–0,5 – 50,0 μg/l dažiklių koncentracijų intervale ($R^2 \geq 0{,}9959$), o aptikimo ribos siekė 0,05–0,18 μg/l. Upės vandens mėginiuose nustatytos 88,6–98,5 % dažiklių priedų išgavos, o rezultatų santykiniai standartiniai nuokrypiai neviršijo 9,6 %.