



Automated on-line dispersive liquid–liquid microextraction based on a sequential injection system

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ABSTRACT

A novel approach for sequential injection-dispersive liquid–liquid microextraction (SI-DLLME) has been suggested. The method is based on the aspiration and mixing of a sample and all required aqueous reagents in the holding coil of an SIA system, delivering it into a conical tube and adding in a mixture of extraction solvent, auxiliary solvent and disperser solvent at high flow rate, resulting in the formation of a cloudy state and the extraction of an analyte. The mixture of extraction and auxiliary solvent is immiscible with water and has a density significantly higher than that of water; consequently, the resulting fine droplets in the mixture, which contain the extracted analyte, are self-sedimented in a short time at the bottom of conical tube. Thus, no centrifugation and no use of a microcolumn are required for separation of the extraction phase. Afterwards, the extracted analyte is aspirated and transferred to a micro-volume Z-flow cell, and the absorbance is measured.

The performance of the suggested approach is demonstrated by the SI-DLLME of thiocyanate ions in the form of ion associate with dimethylindocarbocyanine reagent, followed by spectrophotometric detection. A mixture of amyl acetate (as extraction solvent), tetrachloromethane (as auxiliary solvent) and acetonitrile (as disperser solvent) was selected for the DLLME procedure. The appropriate experimental conditions for conventional DLLME and automated SI-DLLME were investigated. The analytical performance of both these procedures was compared. The absorbance of the colored extracts at wavelength 555 nm obeys Beer's law in the range of 3.13–28.2 for conventional DLLME and 0.29–5.81 mg L⁻¹ of SCN for SI-DLLME, and the limit of detection, calculated from a blank test based on 3 s, is 0.110 for conventional DLLME and 0.017 mg L⁻¹ for SI-DLLME.

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1. Introduction

Despite the undoubted advantages of liquid–liquid extraction (LLE), there was from the mid to late twentieth century a significant decline in the number of publications in the field of conventional LLE after the development of solid phase extraction (SPE) and solid phase microextraction (SPME), although conventional LLE did remain a simple and versatile sample pre-treatment technique in routine analyses. However, from the end of 1990s, new extraction techniques have been developed with the goal of minimizing the known drawbacks of conventional LLE. In recent years two trends have been

observed in the improvement of conventional LLE: (a) miniaturization and (b) automation [1–4].

In the DLLME method, an appropriate mixture of water-immiscible organic solvent as the extraction solvent and water-miscible organic solvent as the disperser solvent is injected rapidly into an aqueous sample solution with a syringe. The resulting cloudy solution is centrifuged, and the fine droplets sedimented in a few micro liters volume at the bottom of a conical test tube are eventually analyzed using chromatography, atomic absorption spectrometry or UV–vis spectrophotometry.

One drawback related to DLLME as it was initially developed in 2006 is that the extraction solvents used must be heavier than water. For extraction solvents lighter than water different systems were suggested [5–11] based on the utilization of special extraction vessels or additional steps for separation. Our research group has very recently suggested an approach based on the use of an auxiliary solvent to adjust the density of the extraction solvent; this method

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offers an alternative to the procedures described in the literature and provides another way of solving the problem of using solvents less dense than water in DLLME [12–16].

Another way of improving conventional LLE is its automation using sequential injection analysis (SIA). This method offers several advantages, such as (a) a closed system for the handling of organic solvent, (b) a lower volume consumption of organic solvent resulting in less organic waste, and (c) a reduced risk of analyte loss and sample contamination.

Despite the important advantages of DLLME, automation of this technique has been a bottleneck until now, probably because DLLME usually requires phase separation by centrifugation. Anthemidis et al. reported an on-line SI-DLLME system coupled to atomic absorption spectrometry in which the organic phase was retained in a microcolumn packed with PTFE-turnings instead of via centrifugation, and a portion of several hundred microliters of isobutylmethylketone was subsequently used for quantitative elution of the analytes, which were then transported directly to the atomic spectrometer [5,17,18]. In our opinion, this solution also has several disadvantages, such as the necessity to use a microcolumn for retention of the analyte as well as the use of several hundred microliters of solvents for elution of the analyte. Moreover, this approach can hardly be applicable for the extraction of ion associates with the subsequent UV–vis spectrophotometric detection that is very often used as a detection technique in sequential injection analysis, since it does not enable separation of excess dye reagent from the ion associate.

The aim of this study was to incorporate dispersive liquid–liquid microextraction into sequential injection analysis. The efficiency of the suggested approach is demonstrated by the DLLME of thiocyanate ions in the form of ion associate with dye reagent, followed by spectrophotometric detection. The suggested approach can also be applied to procedures for the determination of other compounds.

2. Experimental

2.1. Reagents

All chemicals and solvents used were of analytical reagent grade. Ultra pure water from Millipore Milli-Q RG (Millipore, USA) was used throughout the experiment. The thiocyanate stock solution ($1 \times 10^{-2} \text{ mol L}^{-1}$) was prepared by dissolving 0.0381 g NH_4SCN in 50 mL of water. A $1 \times 10^{-2} \text{ mol L}^{-1}$ stock solution of

dimethylindocarbocyanine (DIC) reagent was prepared by dissolving of 0.1965 g in several droplets of methanol and subsequent dilution with water up to a volume of 50 mL. The working solutions of the reagents were prepared by appropriate dilution of the stock solutions. The required pH of the aqueous phase was reached by the addition of HCl of appropriate concentration (in the range of pH 0.2–3) or by using $\text{HOAc-NH}_4\text{OH}$ buffer solution prepared by mixing equimolar (1 mol L^{-1}) solutions of acetic acid and ammonium hydroxide in various volume ratios.

2.2. Apparatus

A Lightwave II UV/Vis spectrophotometer (Biochrom Ltd., United Kingdom) equipped with a matched cell of 1 mm path length was used for UV–vis spectrophotometric measurements. The pH values of the solutions were measured using an ORION 720A⁺ pH meter with a glass electrode. Centrifugation was performed using a CN-2060 LED and Multi-function type centrifuge (MRC Ltd., Israel).

The automated on-line sequential injection dispersive liquid–liquid microextraction (SI-DLLME) manifold (Fig. 1) is based on the commercially available FIALab® 3500 system (FIALab® Instrument Systems Inc., Bellevue, USA), which consists of a 5 mL syringe pump, a central eight-port Cheminert selection valve and a PTFE holding coil (HC_1), and on an external six-port Cheminert selection valve (Valco Instrument Co., Houston, USA), an external 5 mL syringe pump (FIALab® Instrument Systems Inc., Bellevue, USA) and holding coil (HC_2). The extraction was performed in a polypropylene conical tube with a volume of 1.5 mL. An LS-1 tungsten lamp (Ocean Optics Inc., Dunedin, USA) was used as the light source, and a fiber-optic CCD USB 2000 (Ocean Optics Inc., Dunedin, USA) was used as the detector. Data acquisition was performed using FIALab® software.

2.3. Conventional DLLME procedure

1 mL of $1 \times 10^{-3} \text{ mol L}^{-1}$ thiocyanate, 0.5 mL of 0.01 mol L^{-1} HCl and 0.7 mL of $1 \times 10^{-3} \text{ mol L}^{-1}$ DIC were placed in a 10 mL screw-cap glass tube with a conic bottom and thoroughly mixed. The volume was filled up to 5 mL with water. Next, 0.5 mL of a solvents mixture containing amyl acetate as extraction solvent, tetrachloromethane as auxiliary solvent and acetonitrile as disperser solvent in a ratio of 1:1:1.5 (v/v/v) was rapidly injected using a 1 mL glass syringe in order to produce a cloudy state. The mixture was then gently

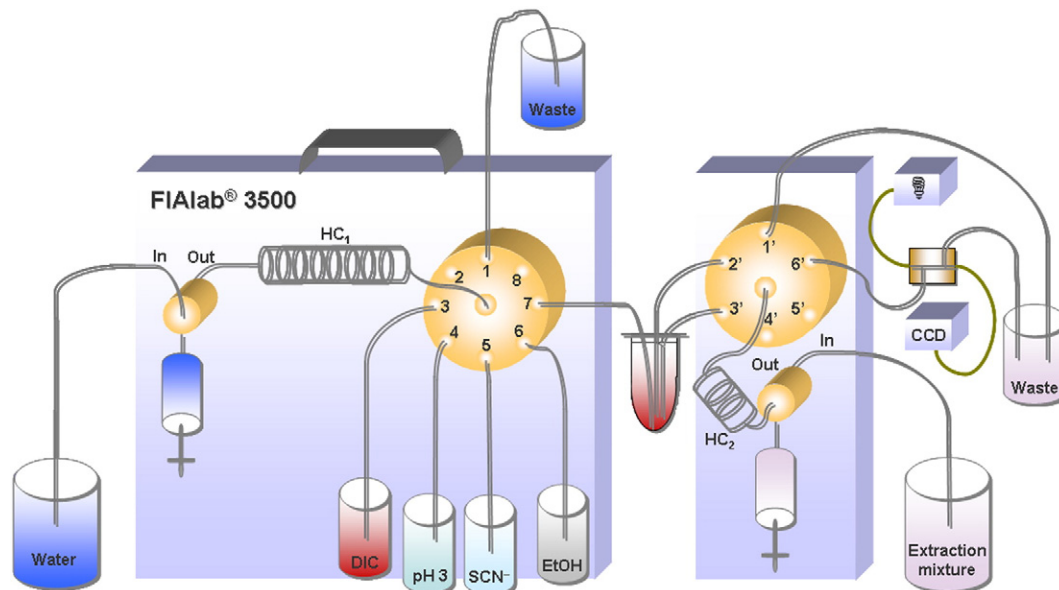


Fig. 1. Automated on-line sequential injection dispersive liquid–liquid microextraction (SI-DLLME) manifold.

shaken 3 times and exposed to centrifugation for 2 min at 3000 rpm. The sedimented phase was withdrawn using a syringe and inserted into the matched quartz cell with 1 mm path length, and the absorbance of the ion associate formed was measured at 555 nm.

2.4. Automated DLLME procedure

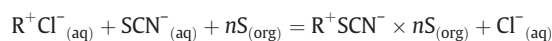
The procedure begins with the syringe pump in the Valve In position, where 655 μL of water is aspirated at $200 \mu\text{L s}^{-1}$. After changing the pump to the Valve Out position, 25 μL of $1.5 \times 10^{-3} \text{ mol L}^{-1}$ DIC solution is aspirated into the HC_1 at $100 \mu\text{L s}^{-1}$, followed by 20 μL of the buffer solution and 100 μL of the standard thiocyanate solution or the sample. Once all the aqueous solutions have been aspirated into the HC_1 , the syringe pump is emptied at $120 \mu\text{L s}^{-1}$, and the aspirated zones are delivered into the conical tube.

Next, the external syringe pump is turned to the Valve In position, and 800 μL of the amyl acetate- CCl_4 -acetonitrile 1:1:2 (v/v/v) mixture is aspirated. This mixture of solvents is used as a carrier and to extract the analyte. The position of the valve is again changed, and 300 μL is dispensed at $350 \mu\text{L s}^{-1}$ into the conical tube containing sample and all the necessary reagents. This step results in a cloudy state, and the analyte is extracted into the fine droplets of extraction solvent formed. The mixture of amyl acetate and CCl_4 is sedimented in a short time at the bottom of conical tube. Then 30 μL of the extracted ion associate is aspirated at $100 \mu\text{L s}^{-1}$ to fill and wash the valve port. The remains of the previously performed extraction (in HC_2) were removed via the auxiliary waste port using 70 μL of the solvents mixture. Finally, 90 μL of the extracted ion associate is again aspirated, transferred into a micro-volume Z-flow cell of 10 mm optical path length by emptying the external syringe pump at $40 \mu\text{L s}^{-1}$, and the absorbance of the colored ion associate is measured at 555 nm.

3. Results and discussion

3.1. Theoretical aspects

The formation and extraction of the R^+SCN^- ion associate are based on ion exchange and may be expressed by the following chemical equation:



From the literature it is apparent that the efficiency of such extractions is mainly determined by the degree of hydration and solvation of the ion associate and the ions that form it. The hydration energy of SCN^- ions (-260 kJ/mol) in various extraction systems is considerably lower than that of Cl^- ions (-331 kJ/mol) [19].

The aim of the calculations was to determine the role and effect of the thiocyanate ion in the DIC^+SCN^- system. Therefore, the DIC^+ cation, the DIC^+Cl^- and the DIC^+SCN^- ion pairs were investigated. For comparison calculations were also carried out for the thiocyanate ion. In this case the B3LYP [20] function was applied within the framework of the DFT method. The basis set was 6-31G*, and the Gaussian 03 program package was used [21]. The optimized geometry, Mulliken atomic net charges and molecular energies were then calculated.

The optimized molecular structures yielded were compared in Figs. S1 and S2. The actual positions of the two DIC nitrogen ions (N_a and N_b) and their positions relative to the chloride ion and the thiocyanate ion (characterized by its nitrogen atom N_f), respectively, are of interest. Table S1 expresses the situations quantitatively. Fig. S1 introduces a planar projection of the three molecules, while Fig. S2 shows, for comparison, the projections of the ion pair structures perpendicular to those in Fig. S1. The figures, as well as Table S1, prove that the thiocyanate ion is closer to the N_a atom than the chloride atom. According to

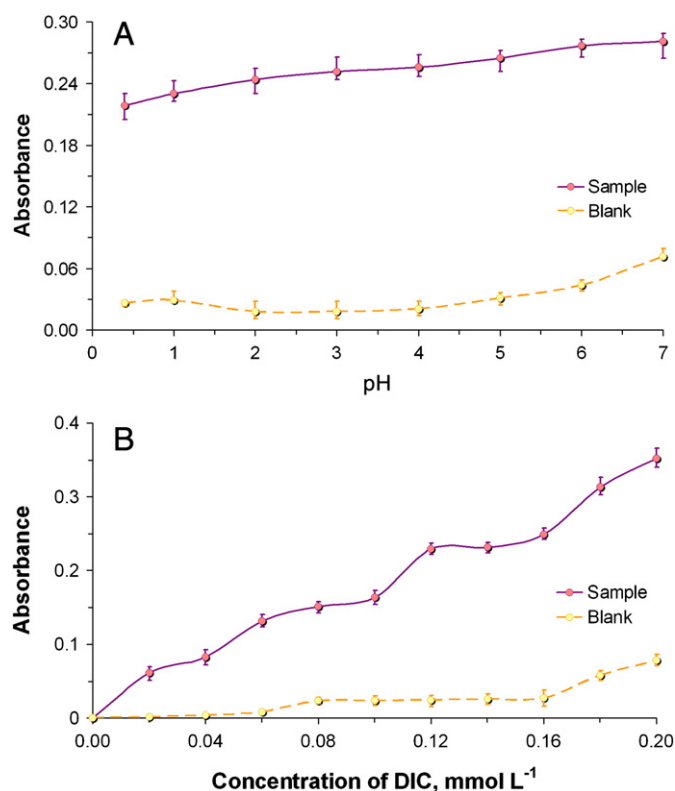


Fig. 2. Investigation of appropriate experimental conditions: A) Effect of pH; B) Effect of DIC concentration $2 \times 10^{-4} \text{ mol L}^{-1}$ thiocyanate; $l = 1 \text{ mm}$; $\lambda = 555 \text{ nm}$; 0.5 mL of amyl acetate/tetrachloromethane/acetonitrile mixture of 1:1:1.5 (v/v/v); A) at 0.14 mmol L^{-1} DIC; B) at pH 3.

Table S1, the N_a - N_b distance depends highly on the presence of the chloride ion and less on the effect of the thiocyanate ion. While the chloride ion could approach the N_a atom to within 5.872 Å, the N_f atom is essentially closer to it, at 3.221 Å.

Table S2 introduces the atomic net charges of some atoms of the studied systems. In contrast to the effects of the anions on the atomic distances, here the effect of the thiocyanate ion on the DIC N_a atom is far stronger than that of the chloride ion. Both ions shift their charge in a positive direction. Their effect on the charge of the N_b atom is a negative shift, but here the effect from the chloride is stronger.

Table S3 presents some characteristic properties of the thiocyanate ion. Comparing the atomic distances of the free anion with the DIC cation, the DIC effect is larger on the SC distance than on the CN distance. The DIC cation has an electron attractive effect on the thiocyanate ion. While the charge on the closer nitrogen atom remains

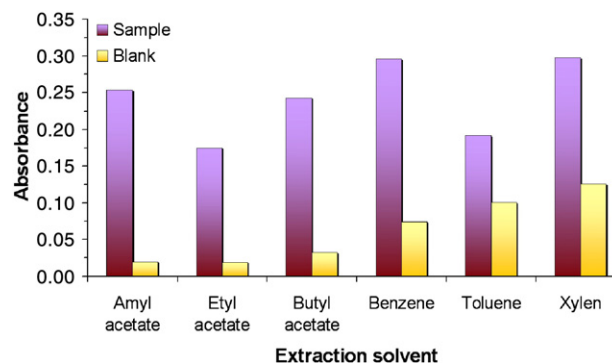


Fig. 3. Effect of extraction solvent $2 \times 10^{-4} \text{ mol L}^{-1}$ thiocyanate; pH 3; 0.14 mmol L^{-1} DIC; $l = 1 \text{ mm}$; 0.5 mL of extraction solvent/tetrachloromethane/acetonitrile mixture 1:1:1.5 (v/v/v).

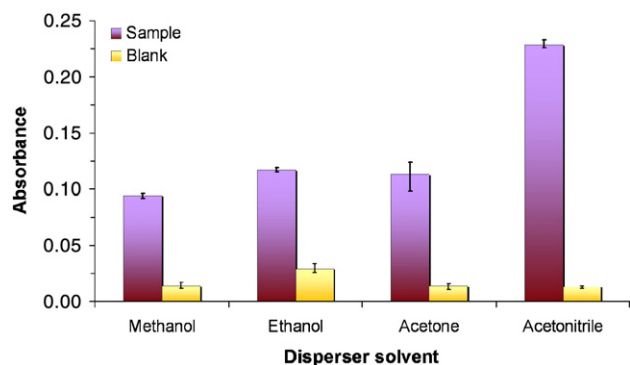


Fig. 4. Effect of disperser solvent 2×10^{-4} mol L⁻¹ thiocyanate; pH 3; 0.14 mmol L⁻¹ DIC; $l = 1$ mm; $\lambda = 555$ nm; 0.5 mL of amyl acetate/tetrachloromethane/disperser solvent mixture 1:1:1.5 (v/v/v).

practically constant, both C and S charges become more positive. This is a reflection of the changes in the distances.

The building energy of DIC^+SCN^- is calculated from the quantum chemical molecular energy results (Table S4). As was expected, the system energy decreases, thus stabilizing the system. One can therefore conclude that the anion change from chloride to thiocyanate causes the deeper penetration of the anion into the structure of the DIC^+ cation. At the same time the cation deforms the thiocyanate anion and acts on its charge distribution.

3.2. Conventional dispersive liquid–liquid microextraction

In order to find the appropriate experimental conditions for formation and extraction of thiocyanate ion associates with DIC, a series of experiments were performed. First, the influence of chemical conditions such as the pH of medium and the concentration of DIC was studied. Then the influence of the nature of solvent and the solvents ratio in the extraction mixture was investigated.

3.2.1. Effect of pH

The effect of the aqueous phase pH on the absorbance of the extracted ion associate was examined in the range from 0.2 to 7 (Fig. 2A). The required pH was adjusted by the addition of HCl (in the range of pH 0.2–3) or 1 mol L⁻¹ HOAc–NH₄OH buffer solution (in the range of pH 3–7). The results showed that extraction is highest in the range of pH 2–5. A pH of 3, which can be achieved using HCl as well buffer solution, was chosen for further studies.

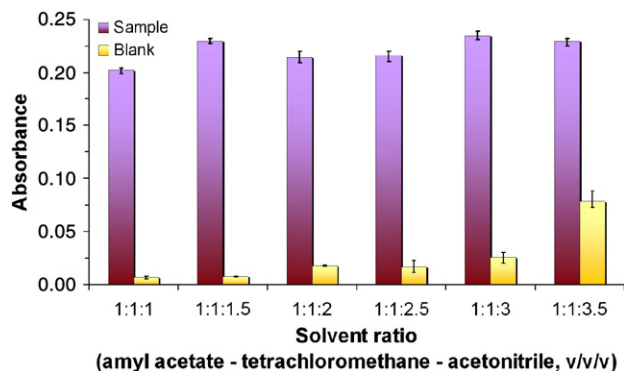


Fig. 5. Effect of solvents ratio in the extraction mixture 2×10^{-4} mol L⁻¹ thiocyanate; pH 3; 0.14 mmol L⁻¹ DIC; $l = 1$ mm; $\lambda = 555$ nm; 0.5 mL of amyl acetate/tetrachloromethane/disperser solvent mixture (v/v/v).

Table 1

Thiocyanate determination in saliva samples using the automated SI-DLLME procedure.

Volunteers (non-smokers)	Thiocyanate, mg g ⁻¹		R, %
	Added	Found	
A	–	0.075	–
	0.040	0.107	90.0
	0.057	0.144	115.7
B	–	0.147	–
	0.045	0.207	110.2
	0.075	0.234	108.2

3.2.2. Effect of DIC concentration

The effect of DIC concentration was investigated in the range 0.02–0.2 mmol L⁻¹ (Fig. 2B). A DIC concentration of 0.14 mmol L⁻¹ was chosen for further studies due to the high value of the analytical signal and simultaneous low absorbance of the blank test.

3.2.3. Effect of the extraction solvent

The main requirement for the extraction solvent is its high efficiency for extracting the target analyte. Usually aromatic hydrocarbons and acetic esters are tested as solvents for the extraction of ion associates. As all of the extraction solvents studied have a density lower than that of the water, it was necessary to use the principle of adjustment of solvents mixture density based on the addition of an auxiliary solvent with a density significantly higher than that of water. This approach was recently developed by our research group [12] and has been applied to the determination of various analytes [13–16]. The main rules for the selection of an auxiliary solvent have been previously discussed in detail [12]. Based on this, tetrachloromethane (density 1.595 g L⁻¹) was chosen as the auxiliary solvent for further studies. The absorption spectra of thiocyanate ion associate with DIC during the extraction by various organic solvents were recorded. The best extractants appear to be benzene, amyl acetate and butyl acetate (Fig. 3). For further experiments, amyl acetate was chosen as the extraction solvent as it has lower toxicity than benzene and lower solubility in water than butyl acetate.

3.2.4. Effect of disperser solvent

In order to find the proper disperser solvent, an extraction mixture containing amyl acetate as the extraction solvent, tetrachloromethane as the auxiliary solvent and various disperser solvents was prepared in a 1:1:1.5 (v/v/v) ratio and was applied to the extraction of thiocyanate ion associate with DIC. Several organic solvents such as methanol, ethanol, acetone, acetonitrile, and DMF were tested as disperser solvents. For choosing the disperser solvent, the value of the analytical signal and the blank test as well as the repeatability of the signal calculated on the basis of three repetitions of the measurements were considered. The obtained results (Fig. 4) showed that acetonitrile is the most suitable disperser solvent.

Table 2

Comparison of conventional and SI-DLLME.

	Conventional DLLME	SI-DLLME
Wavelength, nm	555	555
Path length, mm	1	10
Extraction mixture	Amyl acetate–CCl ₄ –Acetonitrile	
Solvents ratio (v/v/v)	1:1:1.5	1:1:2
Regression equation, mg L ⁻¹		
<i>a</i> (slope)	0.022	0.176
<i>b</i> (intercept)	0.001	0.049
Correlation coefficient, <i>r</i> ²	0.999	0.998
Linear range, mg L ⁻¹	3.13–28.2	0.29–5.81
LOD, mg L ⁻¹	0.110	0.017

3.2.5. Effect of solvents ratio

Solvent ratio in the extraction mixture can noticeably affect the absorbance of ion associate and a blank test. For this reason, a series of experiments were performed in which the ratio of extraction solvent and auxiliary solvent was kept constant at 1:1 (v/v), and the proportion of acetonitrile was varied. With an increase of acetonitrile in the solvents mixture from 1:1:1.5 to 1:1:3.5 (v/v/v), the analytical signal was more or less unaltered (Fig. 5). However, increasing the acetonitrile portion caused a sharp increase in the value of blank test. The highest analytical signal at the lowest value of blank test simultaneous with the best repeatability was obtained at the ratio 1:1:1.5 (v/v/v). This ratio was used in all measurements.

3.3. Automated dispersive liquid–liquid microextraction

3.3.1. Effect of the aspiration sequence and mixing mode

Various aspiration sequences were tested; however, no differences between the sequences were found, so the following order – DIC, buffer solution, and the sample solution – was used in further studies. Similarly, no significant differences were found between the mixing modes (unidirectional and flow reversals) of the reagents and sample in the holding coil before their delivery into the conical tube. Therefore, taking into account the shorter time of analysis, the unidirectional mode was chosen for further investigations.

3.3.2. Effect of the reagents aspirated volumes

The volumes of the DIC and buffer solution were optimized. The volume of the carrier was set in order to keep the total volume transferred to the conical tube constant. Both volumes were studied in 5 μL increments, in ranges from 10 to 30 μL for DIC and 15 to 25 μL for the buffer solution. The volumes of 25 μL of DIC and 20 μL of buffer were selected for further determinations based on the best repeatability of the measurements (lower RSD value).

3.3.3. Optimization of the extraction procedure

To achieve the best efficiency of extraction, three parameters were optimized: the solvent ratio, the flow rate of the organic solvent mixture, and the extraction time – the elapsed time from the moment the solvents mixture was added to the aqueous solution in the conical tube until the organic phase was aspirated and sent to the detector.

3.3.3.1. The effect of the solvent ratio. As in batch conditions, the ratio of the extraction solvent and the auxiliary solvent was kept constant at 1:1 (v/v), and the proportion of the disperser solvent was changed from 1.5 to 4. The increase in the acetonitrile proportion caused an increase in the blank signal, so the best solvent ratio was chosen as a compromise between the highest analytical signal and the lowest blank signal. A 1:1:2 (v/v/v) ratio was picked for further determinations.

One of the features of DLLME is rapid injection of the mixture of solvents into the aqueous phase for the formation of cloudy state. Hence, the flow rate of the solvents mixture was investigated and a rate of 350 $\mu\text{L s}^{-1}$ (maximum flow rate of the auxiliary piston pump) was chosen. Lower flow rate values were not sufficient to produce a cloudy state.

3.3.3.2. The effect of the extraction time. Since the 1:1 v/v mixture of amyl acetate and CCl_4 has a density significantly higher than that of water, the fine droplets in this mixture, which contain the extracted analyte in the form of ion associate with DIC, sediment in short time at the bottom of conical tube. Therefore, 3 s was selected for further experiments – the minimum time needed for self-separation of the phases.

3.3.4. Analytical futures

A calibration graph was constructed from seven data points over the range of 0.29–5.81 mg L^{-1} . The regression equation was

$$A = (0.176 \pm 0.005) [\text{SCN}^- \text{mg L}^{-1}] + (0.049 \pm 0.015)$$

with a correlation coefficient of 0.998. The LOD calculated from the calibration plot based on 3s was 0.017 mg L^{-1} . The sample throughput was 8 h^{-1} for samples aspirated in triplicate and including the washing step. The intra-day precision and the inter-day precision were examined using RSD (%) values. Intra-day precision values for two concentration levels: 1.45 and 2.90 mg L^{-1} were 5.3% and 6.5%, respectively. Inter-day precision was evaluated measuring the same concentrations in triplicate over 4 days, and the values were 4.7 and 9.4%, respectively.

3.3.5. Analytical application

Human saliva is composed of 98% water and, in minor percentage, of macromolecules, antibacterial compounds and inorganic ions such as thiocyanate. The concentration of SCN^- ions in saliva can vary as a result of the digestion of glucosinolate-containing vegetables or by the intake of thiocyanate-containing food [22,23]. Drugs used in the treatment of thyroid glands diseases and arterial hypertension can also affect SCN^- quantity. As this ion is a metabolic product of cyanide, a person who smokes has a higher concentration of thiocyanate in saliva.

The developed automated on-line DLLME procedure was applied for determination of thiocyanate ions in saliva samples. About 1 mL of saliva samples was collected in previously weighted conical plastic tubes from two healthy volunteers (females, non-smokers) between the breakfast and the lunchtime. After the sample was taken, the tubes were reweighed to calculate the quantity of sample to be analyzed. Afterwards, the sample was diluted to 10 mL with water and centrifuged for 10 min at 5000 rpm. The supernatant solution was diluted 4-fold with water and 100 μL were then aspirated by the SIA system in order to perform the analysis. To carry out the recovery study, the samples were spiked with 60 and 100 μL of $1 \times 10^{-2} \text{ mol L}^{-1} \text{ NH}_4\text{SCN}$ solution, and the same protocol was followed. The added amounts of standard solution were recalculated with consideration of the sample weight. The concentrations of SCN^- ions, expressed as milligrams of SCN^- per gram of saliva sample and the recoveries are shown in Table 1. The obtained values were in good agreement with those found in the literature [23], and the recovery study also showed satisfactory results.

4. Comparison of conventional and automated DLLME method

The analytical parameters of conventional and automated DLLME were compared. As can be seen in Table 2, the automated method presented a linear range for lower concentrations and an LOD value lower by one order of magnitude, meaning that it is possible to determine lower concentrations of thiocyanate ions. Sample consumption for the automated method was significantly less than for the conventional one, which is of highlighted importance when the available quantity of sample is limited. Moreover, automation offers great advantages for the operator, etc.

5. Conclusion

A novel approach for automated on-line sequential injection-dispersive liquid–liquid microextraction (SI-DLLME) has been suggested which requires no centrifugation and no use of a microcolumn for separation of the extraction phase. The instrumentation is based only on commercially available components. The analytical performance of the developed automated SI-DLLME procedure was compared with that of conventional and automated DLLME, and the procedure was applied for determination of thiocyanate ions in saliva samples. The suggested approach can also be applied to procedures for the determination of other compounds which can be extracted using organic solvents.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.microc.2011.09.006.

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