CHEMIJA. 2009. Vol. 20. No. 3. P. 180–185

Determination of low-residue benzodiazepine, lorazepam, in environmental water samples by suspended droplet microextraction and high performance liquid chromatography-diod array detector

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Three-phase liquid-phase microextraction (LLLME) was developed for high performance liquid chromatography and capillary electrophoresis. The new branch of this mode, entitled suspended droplet liquid phase microextraction (SD-LPME), involves the use of a suspended droplet of an aqueous solvent. In this technique, the droplet is floated freely in the surface-center of an immiscible organic solvent, which has been laid on the surface of the aqueous sample while being agitated by a stirring bar. The performance of this technique is demonstrated in the determination of one of benzodiazepines, lorazepam, which is extracted into a single drop of aqueous solution. SD-LPME has provided good enrichment (645-fold), but relatively poor reproducibility: RSD%: 5.04, n = 5 (primarily due to repeated manual manipulation), simplicity, relatively fast extraction and back-extraction times (30.0 and 60.0 seconds, respectively). This method has allowed a direct transfer of the extracted analyte into a high performance liquid chromatography-diod array detector (HPLC-DAD).

Key words: HPLC-DAD, suspended droplet, liquid phase microextraction, lorazepam, aqueous samples

INTRODUCTION

The group of 1,4-benzodiazepines is widely used in the treatment of nervous diseases such as anxiety, insomnia, muscle spasms and seizures. Many patients develop a dependence on these drugs which are often involved in intoxications. Consequently, benzodiazepines are frequently encountered in clinical, forensic, toxicological and water sample analysis [1]. Lorazepam (7-chloro-5-(2-chlorophenyl)-3hydroxy-2,3dihydro-2H-1,4benzodiazepin-2-one) is one of the 1,4-benzodiazepine drivatives that are most widely used [2].

Many analytical methods have been published for the determination of benzodiazepines. Most of these methods can be applied to the parent benzodiazepine and its corresponding metabolites. Traditional liquid–liquid extraction (LLE) is still a very popular technique [3, 10, 11, 17, 32]. This technique provides analyte enrichment by a factor of 2–10. Evaporation of the solvent to dryness and reconstitution of the dry residue in a smaller solvent volume may lead to further enrichment. Evaporation of solvent and reconstitution are time-consuming processes which utilize relatively large amounts of solvents. These processes could be avoided if the sample preparation method delivered the target analytes in a sufficiently small volume of solvent, suitable for direct injection into an analytical instrument. Liquid phase microextraction is a sample preparation technique eligible for small sample volumes [13].

Recently, in the field of liquid–liquid microextraction, Yangcheng and coworkers developed a new sampling method termed directly suspended droplet microextraction (DSDME) [34]. In this research, a new application of DSDME, i. e. three-phase DSDME which is appicable for coupling to HPLC, was used for extraction of lorazepam from real water samples. Three-phase microextraction, and consequently a three-phase DSDME technique, was developed to extract ionisable and chargeable compounds from different aqueous samples [14, 15]. In three-phase DSDME, a droplet of an aqueous solvent is suspended freely in the surface-center of an immiscible organic solvent, which has been laid on the surface of the aqueous sample while being agitated by a stir

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bar. Agitation of the sample causes a weak gentle vortex. If a small volume of an aqueous droplet is added to the surface of the organic solvent, the vortex results in the formation of a single microdroplet at or near the center of rotation. The droplet itself also rotates on the surface of the organic solvent, so mass transfer is increased. Compared with the conventional LPME technique based on the droplet system, i. e. single drop microextraction (SDME), it provides more flexibility in the choice of the operational parameters, especially as regards solvent dosage and stirring frequency [29, 34]. In this research, the pH of the donor phase (aqueous solution of lorazepam) was basic and the acceptor phase was acidic. The analyte was extracted from the donor phase into the organic phase, then back-extracted into the acceptor phase [14,1 5, 28, 35].

Several chromatographic procedures have been described for the analysis of lorazepam [3, 16, 19, 22]. The gas chromatographic method appears to be more sensitive than high-performance liquid chromatography; however, it requires sample derivatization for drug analysis, thus increasing the complexity and time required for analysis. In addition, thermal instability of this compound at the high column temperature used in gas chromatography complicates the analysis [6, 10]. In the present work, we report HPLC-DAD analysis after the above-mentioned microextraction technique.

EXPERIMENTAL

Chemicals, reagents and standards

HPLC-grade organic solvents; methanol, acetonitrile, 1-octanol, n-hexane, benzyl- alcohol, toluene, dichloromethane were obtained from Merck (Darmstadt, Germany). Lorazepam was a gift from Loghmanpharmd Co,Iran. Deionized water was purified in a Milli-Q purification system (Millipore). The stock solution of lorazepam (500 mg l^{-1}) was prepared by dissolving a calculated amount of lorazepam in methanol, and it was stored at 4 °C. Standard sample solutions containing the target compound were provided daily at different concentrations by diluting the stock standard solutions with triple-distilled water. The used water was purified on a Milli-Q ultra-pure water purification system (Millipore, Bedford, MA, USA).

HPLC system

The separation, identification and quantification were carried out on a KNAVER HPLC system, HPLC: KNAVER Jahre35 (Germany). The chromatography was performed isocratically on a C18, MZ-Analytical column (250 × 4.6 mm), Perfectsil Targetat, ODS-3 5 μm. A RP-18 guard column was fitted upstream of the analytical column. The mobile phase was acetonitrile–methanol–water optimized on (20–5–75%, pH 5) and was delivered by a KNAVER K-1001HPLC pump. The flow rate of the mobile phase was 1 ml min^{-1}, and a diode array detector K-2800 was used for its detection. The elution was monitored at 200 nm. The other equipment included a KNAVER K-1500 solvent organizer, KNAVER K-500 degasser, a sono bath (LIARRE-Italy), a pH meter 744 (Metrohm, Switzerland), HPLC syringe 10, 25 μl (Hamilton, Switzerland). The system was equipped with Eurochrom HPLC software, version 3.05.

Directly suspended droplet microextraction procedure

The sample solution (4.5 ml, adjusted to pH 13 with NaOH) was placed in a 5 ml glass vial. A stirring bar (7 × 3 mm) was used to facilitate the mass transfer process. A magnetic stirrer (0–1000 rpm) was used to stir the extraction mixture. A 10 μl flat-cut HPLC microsyringe (Hamilton, Bonaduz AG, Bonaduz, Switzerland) was used to introduce the acceptor phase and acted as an injection syringe. The experimental microextraction setup is shown in Fig. 1. Extractions were performed according to the following procedure. The sample solution was added to a glass vial, and a magnetic bar was placed into the vial; 400 μl of organic solvent was then added to the sample solution with a microsyringe. Then the mixture was agitated for 30 s at 1000 rpm. After this time, the acceptor phase (10 μl aqueous droplet, pH 3) was delivered to the top-center position of the immiscible organic solvent. The mixture was agitated at 300 rpm for 60 s, the microdroplet was withdrawn into a syringe and injected to HPLC for analysis.

RESULTS AND DISCUSSION

Optimization of directly suspended droplet microextraction

The different parameters that influence the extraction were optimized. The optimization was carried out on a water solution of 2 mg l^{-1} lorazepam. The parameters such as the kind of organic solvent, extraction time, microdroplet volume, stirring rate and pH were considered and optimized.

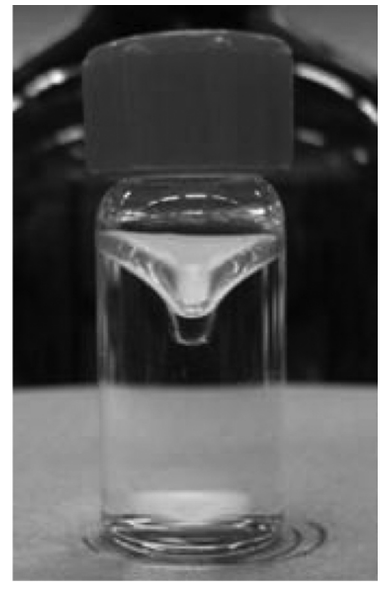

Fig. 1. A Photograph of the DSDME process

Choice of organic solvent

It is necessary to choose a convenient organic solvent, which depends on the chemical nature of the target compounds, for the establishment of a direct mode LPME technique. The choice of the organic solvent needs the following considerations. The solvent should have a good affinity for the target compounds, a low solubility in water so as to prevent the dissolution in the aqueous phase, and a lower density than water [28, 29]. On the basis of these considerations, 1-octanol, benzylalcohol-octanol (30 : 70), n-hexan, toluene, hexan-dicholoromethan (30 : 70) were tested, but the aqueous droplet was only stable in 1-octanol. Therefore, 1-octanol was used as the extraction solvent for the further extraction procedure.

Phase volumes

The enrichment factor can be improved by increasing the volume ratio of donor and acceptor phases [20, 23, 31]. The best extraction efficiency was obtained when the donor–acceptor ratio was more than 100. Furthermore, the volume of the acceptor solution used for extraction may also be adjusted depending on the analytical technique coupled to LLLME. For example, compared with GC, the injected sample volume in HPLC may be in the range of 10–25 µl. Therefore, the whole acceptor phase can be analysed and a lower detection limit obtained [20]. In this manner, the use of a larger drop results in an increase of the analytical response, but these large drops are not very stable, especially with a high stirring speed, and may fall into the sample solution (donor phase). Thus, a 10 µl droplet was chosen as the optimum volume of acceptor phase. On the other hand, because of the design of our extraction device, the volume of the organic phase was also important and needed to be optimized. The best volume of the organic solvent was found to be 400 µl. A smaller volume of organic solvent (i. e. less than 300 µl) tends to cause instability of the aqueous drop during agitation, whereas the extraction efficiency is reduced if a larger volume of organic phase is used. Therefore, a 400 µl volume of organic solvent was chosen for the subsequent work.

Extraction time (T1)

The extraction of the analyte from the water sample (P_1) into the organic phase by LLLME is a slow equilibrium process, and mass transfer is time-dependent [18]. Because solute molecules need sufficient time to pass the interface between the donor and organic phases, the recovery depends on the time when the analyte is in contact with the organic phase. The extraction time is one of the most important factors influencing the extraction efficiency. Before addition of the suspended aqueous droplet, the mixed aqueous and organic solution was agitated at 1000 rpm for 30 s (T_1) to give a cloudy mixture of the sample solution and organic solvent. Due to the high degree of mixing between the donor and organic phases, the mass transfer is rapid [30].

Back-extraction time (T2)

Three-phase suspended droplet is not an exhaustive extraction technique. Although the maximum efficiency is attained at equilibrium, a complete equilibrium is not necessary because of increasing the analysis time [9, 18]. Droplet lifetime cannot be too long due to drop dissolution or loss. Therefore, the back-extraction time (T2) from the organic solvent (1-octanol) into the aqueous acceptor phase (10 µl, pH 3) should not be too long, and 60 s was chosen (Fig. 2).

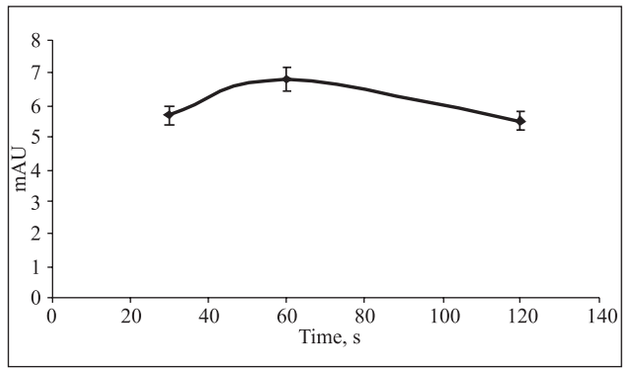

Fig. 2. Effect of back extraction time on the extraction. Experimental conditions are as follows: donor phase volume, 4.50 ml; acceptor phase volume, 10.0 µl (phase ratio = 450); donor phase pH, 11.0; acceptor phase pH, 3.0; stirring rate, 200 rpm

Stirring speed

Agitation of the sample solution is generally applied to facilitate the mass transfer process and accelerate the extraction kinetics. Increasing the stirring speed of the donor phase enhances the diffusion of analyte through the organic phase and improves the repeatability of extraction [25, 26]. Therefore, the stirring speed was also optimized for a better extraction. Different stirring rates (100, 300, 500 rpm) were checked (Fig. 3). In order to speed up extractions, a stir bar was added to each sample, and stirring was conducted at 300 rpm. A reduction in the efficiency after the stirring speed reached its maximum value (300 rpm) was observed for the analyte. It is possible that at much higher flow rates, due to the high velocity of the sample solution, the suspended droplet was unstable. Moreover, establishing the extraction equilibrium in the interfacial layer of both phases is difficult.

The pH of acceptor and donor phases

The pH of both donor (sample solution) and acceptor phases affects the extraction performance. For basic drugs, the donor phase should be strongly alkalized to effectively deionize the analytes and consequently reduce their solubility within the sample, while the acceptor phase should be acidized in order to promote dissolution of the basic analytes [4, 5, 21, 33]. The effects of sample pH in the range of 7–13 was investigated (Fig. 4). As a result, the best extraction efficiency was observed at pH 13.

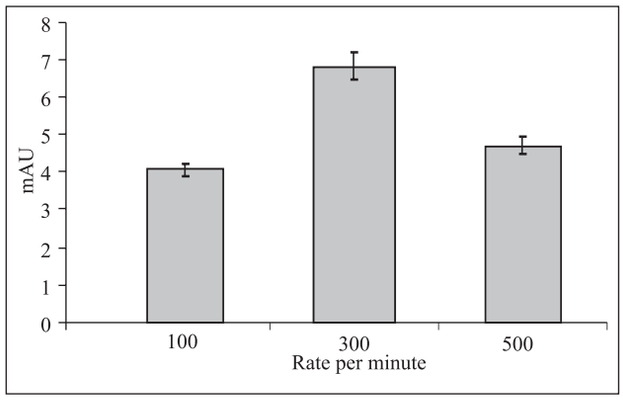

Fig. 3. Effect of stirring speed on the extraction procedure. Experimental conditions are as follows: donor phase volume, 4.50 ml; acceptor phase volume, 10.0 µl (phase ratio = 450); donor phase pH, 11.0; acceptor phase pH, 3.0; back extraction time, 60.0 seconds

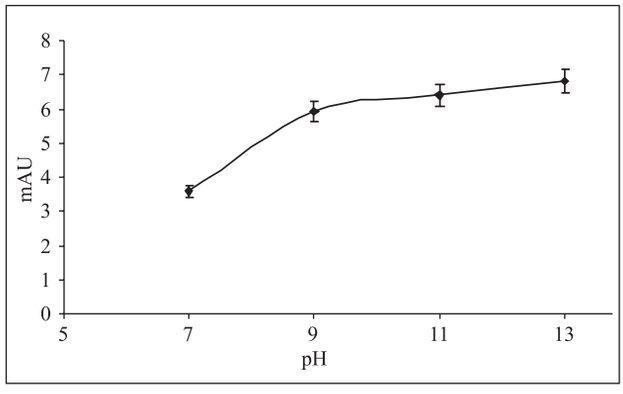

Fig. 4. Effect of pH on the DSDME. Experimental conditions are as follows: donor phase volume, 4.50 ml; acceptor phase volume, 10.0 µl (phase ratio = 450); donor phase pH, 13.0; acceptor phase pH, 3.0; back extraction time, 60.0 seconds

Therefore, pH 13 of the donor phase was selected, and the pH of the acceptor phase was fixed at pH 3.

Salt effect and ionic strength

In the LPME, the 'salting-out' of relatively water-soluble analytes is possible. This relies on increasing the ionic strength of the aqueous phase, thus encouraging partition of a relatively water-soluble analyte into an immiscible organic solvent. The common substances used for this purpose include ammonium chloride, ammonium sulfate, sodium chloride, sodium citrate, sodium sulfate and potassium carbonate. However, with LLE, caution is needed because an emulsion may form if excess salt is added. It was noticed that in our research, the presence of salt had no positive effect on the extraction efficiency at all concentration levels of sodium chloride. Not only this behaviour, but also decreasing effects were reported by others [2, 24], which may be due to several reasons; the NaCl dissolved in water might have changed the physical properties of the Nernst diffusion film and reduce the rate of diffusion of target analytes into the microdrop [27]. On the other hand, with NaCl addition, the viscosity of the bulk solution increases, and the diffusion rate of the analytes from the bulk solution to the organic phase is affected [12]. Therefore, other measurements were carried out without salt addition.

Quantitative analysis of lorazepam

The calibration graph for the determination of lorazepam in water was based on the peak area (Fig. 5). The enrichment factor, linear range, precision (RSD) and the limit of detection (LOD) were calculated under optimum experimental conditions.

The concentration enrichment (EF) was calculated by the following formula [20, 21, 23–26, 28, 29]:

$$EF = (C_a, final) / (C_s, initial) = (V_s / V_a) \times R / 100.$$

The enrichment factor is 645, and the linearity of this method for analysing a standard solution has been investigated in the range 30–5500 ng ml^{-1}. The precision of the method (RSD%) is 5.04 based on peak areas for five replicates. The limit of detection of 25 ng ml^{-1} is based on a signal-to-noise ratio of 3, calculated for five replicate runs (Table 1).

Real water analysis

To demonstrate the practical applicability of the new technique, real water samples were analysed by this method. Drinking water from the Mashhad water-supply network, industrial wastewater and clinical wastewater were spiked with 50 ng ml^{-1}of lorazepam and extracted under optimal conditions. The results are shown in Fig. 6 and Table 2.

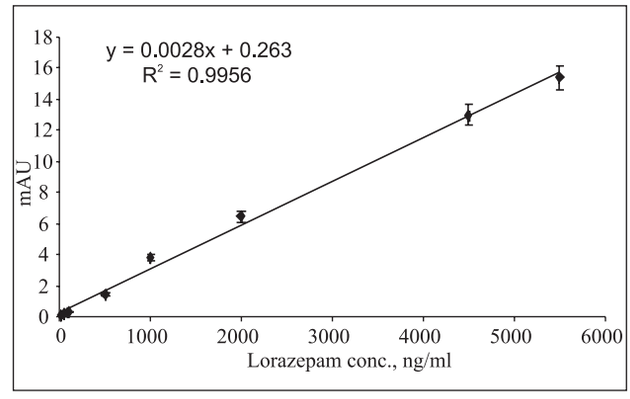

Fig. 5. The calibration graph for lorazepam under optimal conditions

Table 1. **SDME performance and validation data**

Analyte	EF	RSD %	LOD (ng ml^{-1})	LOQ (ng ml^{-1})	Linear range (ng ml^{-1})	R^2	Equation
Lorazepam	645	5.04	25	30	30–5500	0.9956	Y = 0.0028X + 0.263

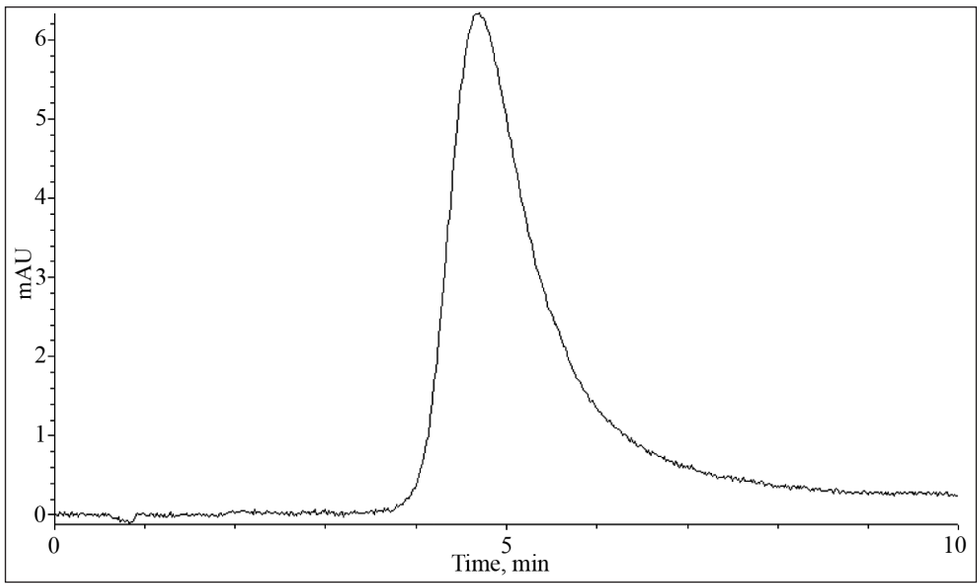

Fig. 6. Chromatograms of Mashhad water-supply network siked with 50 ng ml^{-1} of Lorazepam and extracted under optimal conditions

Table 2. **Relative recoveries and precisions of SDME technique for real waters spiked with the analyte**

Water sample	RR%	RSD%
Tap water	97.4	7.7
Industrial wastewater	98.6	8.4
Clinical wastewater	99.9	7.8

SD-LPME is not an exhaustive extraction method, so relative recovery was determined as the ratio of the concentration found in real samples and distilled water sample, with both samples spiked at the same concentration level under optimized conditions [36].

The relative recovery of the analyte from all of these real water samples was higher than 89%, indicating that the matrix effect does not have any significant effect on the extraction efficiency.

CONCLUSIONS

A new, simple, rapid and efficient method of liquid phase microextraction, referred to as directly suspended droplet liquid–liquid–liquid phase microextraction, has been developed. Compared to most conventional procedures, this extraction technique requires very little sample solution as well as little expensive and toxic organic solvents. It is a promising pre-treatment method for a fast, trace analysis in many complicated matrices, such as environmental and biological samples. The method has a high enrichment factor and an excellent selective cleanup of samples. A good linearity and a reasonable relative recovery were also obtained. We used the method to isolate lorazepam from natural water samples and found it to have many advantages over the conventional LPME. The method was compared with many techniques used for determination of lorazepam in environmental and biological samples, and the results was shown in Table 3. One can see that comparable results were achieved for this method versus conventional microextraction methods and without the use of advanced instruments such as LC–MS, LC–MS–MS or GC–MS.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the Islamic Azad University of Mashhad, Iran for financial support of this work.

Received 8 May 2009
Accepted 18 May 2009

Table 3. **Comparison of the SDME-HPLC-DAD with other related methods of lorazepam determination**

Method	Matrix	LOD	RSD %	Recovery %
LLE / HPLC [1]	Plasma	0.781 μg l^{-1}	–	97.2–103
SPE / GC–MS [22]	Plasma, urine	0.1 ng ml^{-1} LOQ)	–	–
SPE / GC–MS [3]	Urine	1.6 ng ml^{-1}	3.6–11.05	58.87–64.71
LLE / LC–MS–MS [11]	Urine, hair, oral fluid	0.5 pg mg^{-1}	<12	–
LLE / HPLC [17]	Plasma	2.5 ng ml^{-1}	7.8–9.8	72.4–84.1
LLE / LC–MS [32]	Hair	0.5–5 pg mg^{-1} (LOQ)	–	32–76
SPE / LC–MS–MS [16]	Urine	≤0.05 μg ml^{-1}	–	77–110
LLE / HPLC [10]	Serum	1 ng ml^{-1}	3.6–5.4	93.9–98.8
SPE / LC–MS–MS [19]	Urine, plasma	0.02–0.15 ng ml^{-1}	–	–
DSDME / HPLC [this research]	Water samples	25 ng ml^{-1}	5.04	97.4–99.9

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BENZODIAZEPINO DARINIO LORAZEPAMO MAŽŲ KIEKIŲ NUSTATYMAS APLINKOS VANDENS PAVYZDŽIUOSE NAUDOJANT KABANČIO LAŠO MIKROEKSTRAKCIJĄ IR DIDELIO NAŠUMO SKYSČIŲ CHROMATOGRAFIJĄ SU DIODŲ MATRICOS DETEKCIJA

S a n t r a u k a

Sukurtas trijų skysčio fazių mikroekstrakcijos būdas didelio našumo skysčių chromatografijai ir kapiliarinei elektroforezei. Naujoje šio būdo modifikacijoje, pavadintoje kabančio lašo skystafaze mikroekstrakcija, naudojamas kabantis vandens lašas, kuris plūduriuoja nesimaišančio organinio tirpiklio paviršiaus centre, o organinis tirpiklis dengia nuolat maišomą tiriamojo pavyzdžio vandeninį tirpalą. Siūlomas būdas taikytas benzodiazepino dariniui lorazepamui nustatyti. Jis pasižymi dideliu įsodrinimo laipsniu (645 kartų) ir santykinai mažu atsikartojamumu (standartinis nuokrypis 5,04 %, n = 5). Būdas yra paprastas ir santykinai spartus; ekstrakcija ir atgalinė ekstrakcija apytikriai trunka atitinkamai 30 ir 60 s.