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Ultrasound and Vortex-Assisted Dispersive Liquid-Liquid Microextraction of Parabens From Personal Care Products and Urine Followed By High Performance Liquid Chromatography

Short Title: Microextraction of Parabens

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Abstract

Objectives: Parabens, which are p-hydroxybenzoic acid esters, are utilized as preservatives in personal care products, pharmaceuticals and food because of their antimicrobial activity. However, they are also

classified as suspected endocrine disruptors and suspected carcinogens. In the present study, it was aimed to optimize an ultrasound and vortex-assisted dispersive liquid-liquid microextraction procedure for simultaneous extraction of methyl, ethyl, isopropyl, propyl, isobutyl and butyl parabens from personal care products and urine.

Materials and methods: The parameters as extraction solvent type, extraction solvent volume, disperser solvent volume, sodium chloride concentration, ultrasonication time and vortex application time were evaluated to obtain optimum recoveries by vortex-assisted dispersive liquid-liquid microextraction. Parabens were detected by a validated high performance liquid chromatography method with fluorescence detection. The method validation was performed by examining linearity, limit of detection, limit of quantification, accuracy and precision.

Results: Limit of detection and limit of quantification of the high performance liquid chromatography method were between 0.09-0.18 µg/mL and 0.28-0.54 µg/mL, rspectively. Precision was examined as relative standard deviation which was in the range of 0.22-1.81% and 1.12-2.03% for intra- and interday studies. Recovery percentages were higher than 96.00%. Samples of two paraben-free personal care products and a synthetic urine were spiked with the analytes at 0.02 µg/mL and were successfully analyzed by the developed procedure with recovery values higher than 82.00%.

Conclusion: The proposed procedure provided quantification of selected parabens at 20 ng/mL level in analyzed personal care product and urine matrices with good precision and accuracy.

Keywords: high performance liquid chromatography; liquid-liquid microextraction; paraben; personal care product; urine

Introduction

Parabens, which are p-hydroxy benzoic acid esters, are widely utilized in various types of food, pharmaceuticals and personal care products as preservatives due to their antimicrobial activity within a wide pH range, high stability, water solubility and low cost. Among them methylparaben (MP), ethylparaben (EP), propylparaben (PP) and butylparaben (BP) are the mostly used ones individually or as mixtures.[1,2] However recent studies have shown the affinity of parabens for binding to the estrogen.[3,4] Their estrogenic effect was assumed to be able to cause breast cancer.[5,6] In addition, some negative impacts on the male reproductive system were reported.[7] In the light of the related researches, parabens are classified as suspected endocrine disruptors and suspected carcinogens.

One of the ways of the exposure to the parabens is the personal care products containing various types of them as preservatives since parabens are absorbed through the skin.[8] The maximum permitted level for PP and BP is 0.14% when used individually or together with other esters in cosmetics by European Commission. The use of isopropylparaben (IPP), isobutylparaben, phenylparaben, benzylparaben and pentylparaben were restricted.[9]

The reliable analysis of parabens has become an issue of great scientific interest because of their suspected damages to human health. Various types of pre-treatment techniques were developed for the pre-concentration or extraction of parabens considering low concentrations and complex sample matrices.[8,10,11] Among them dispersive liquid-liquid microextraction (DLLME) is a common technique with advantages including requirement of lower extraction solvent volume, lower sample amount and being less time consuming comparing with the traditional procedures. The extraction solvent is immisicible with the sample solution and the disperser solvent is used to obtain a better contact between them.[12] In a study, MP, EP, PP and BP were successfully extracted by DLLME from breast milk.[8] Oliveira et al. (2019) also determined 17 potential endocrine disrupting chemicals including MP, EP, PP, BP and benzylparaben by DLLME coupled with LC-MS/MS in human saliva.[13] In another study DLLME of MP, EP, PP, BP from pharmaceuticals and personal care products were performed.[14]

Parabens are generally extracted from various matrices using chlorinated solvents which may affect human health negatively and are not environmentally friendly.[13,14] A special technique of DLLME, called as ultrasound and vortex-assisted DLLME (USVADLLME), was developed by which the required volume of those hazardous extraction solvents was reduced. In USVADLLME procedure, the ultrasonication provides a better dispersion and vortex prevents biphasic system formation.[15]

High performance liquid chromatography (HPLC)[16,17] and gas chromatography (GC)[18,19] are two common detection methods of parabens in different types of sample matrices as food products[20], biological fluids[16,17,21], environmental samples[22,23], pharmaceuticals[24,25] and personal care products[26,27]. Among them GC methods may require steps of derivatization or pre-concentration. HPLC with ultraviolet (UV) or diode array detection has disadvantges such as the interference of the other ingredients and high detection limits. Liquid chromatography with mass spectrometry (LC-MS) or with tandem mass spectrometry (LC-MS/MS) may avoid all of these drawbacks, while these systems are unavailable in many laboratories because of their high costs. On the other hand, HPLC with fluorescence detection (FD) may also be used which has higher selectivity than UV detection and is more available than MS systems. An HPLC-FD method was developed, validated and applied successfully for four types of parabens as MP, EP, PP and BP in cosmetic products in a recent work.[28] In addition Yılmaz et al. (2020) developed a method MP, EP, PP, isobutylparaben (IBP) and benzyl paraben (BzP) by HPLC-FD in cosmetics.[29]

In the present study, it was aimed to optimize an USVADLLME procedure for the extraction of six parabens (MP, EP, IPP, PP, IBP and BP) (Fig. 1) from personal care products and synthetic urine. For the quantification of the extracted parabens a HPLC-FD method was developed and validated according to the parameters as linearity, limits of detection (LOD) and quantification (LOQ), accuracy and precision considering the advantages of FD mentioned above. The proposed USVADLLME technique has never been used for the extraction of the selected analytes. To our best knowledge the proposed work could be the first USVADLLME coupled with HPLC-FD method on detection of parabens in personal care products and urine.

Materials and Methods

Reagents and chemicals

Standard materials of MP, EP, IPP, PP, IBP, BP and the synthetic urine (Surine™ Negative Urine Control) were purchased from Sigma (Darmstadt, Germany). The HPLC grade methanol (MeOH), o-phosphoric acid, sodium chloride (NaCl) dichloromethane (CH_2Cl_2) and chloroform ($CHCl_3$) were from Merck (Darmstadt, Germany). The chemicals were analytical grade.

A stock solution at 100.00 µg/mL for each of the analytes was prepared with MeOH (HPLC grade) and stored at 4°C. The stock solution was diluted daily with the mobile phase to obtain the standard paraben solutions at desired concentrations.

The paraben-free tonic sample (T) and the paraben-free micellar water sample (MW) were from a commercial supplier in Istanbul, Turkey (2018).

HPLC conditions

The analysis of the parabens were performed by an HPLC system (LC20AT, Shimadzu, Kyoto, Japan) with fluorescence detection (RF20A). Analytes were separated by a C18 analytical column (4.6 × 250 mm, 5.0 µm) (Intersil ODS-3, GL Sciences Inc., Tokyo, Japan). The mobile phase system was consisted of 50% phosphate buffer (0.1 M, pH 7) and 50% MeOH. Isocratic elution was applied at 1.0 mL/min. The column temperature was 40°C. The excitation and the emission wavelengths were 254 and 310 nm, respectively. The injection volume was set to 20 µL. Data analyses were evaluated by LabSolutions software (version 1.25).

USVADLLME procedure

A hundred and fifty microliters of $CHCl_3$ and 50 µL of MeOH were transferred into a conical-bottom glass test tube with a screw cap which contained 5 mL of sample solution. Then, NaCl was added (2.0 g/L). The solution was vortexed (VTX-3000L, Harmony, Tokyo, Japan) for 4 minutes and ultrasonicated

for 90 seconds (Elma Hans Schmidbauer GmbH & Co. KG, Siegen, Germany). Following ultrasonication, the solution was centrifugated for 3 minutes at 4000 rpm (VWR Compactstar CS4, VWR International Ltd**,** Leicestershire, UK). A microsyringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) was used to separate the $CHCl_3$ phase, which was then evaporated under N_2 flow. Residue was dissolved in 100 µL of the mobile phase prior to HPLC analysis. The extraction procedure was applied in triplicate for all of the analyzed samples.

Results and Discussion

Selection of HPLC conditions

Various mobile phase types were examined using MeOH, acetonitrile, acetic acid, formic acid and phosphate buffers for the suitable separation of the parabens. A mobile phase system consisted of 50% phosphate buffer (0.1 M, pH 7) and 50% MeOH with isocratic elution was selected considering the parameters of baseline drift, retention time and resolution. The excitation wavelength of 254 nm and the emission wavelength of 310 nm were selected to obtain optimum signals for all of the analytes.

Method validation

Validation of the proposed HPLC-FD method was evaluated with the parameters of linearity, limits of detection (LOD) and quantification (LOQ), precision and accuracy. A representative chromatogram of the analytes (2.50 µg/mL) was given in Fig. 2.

A 6-point calibration curve was prepared for each of the parabens (0.50-10.00 µg /mL). The linearity was examined by regression results. Suitable linearities were obtained for all of the analytes ($r > 0.99$) (Table 1).

LODs were calculated as 3.3 times, whereas LOQs were determined as 10 times of the ratio of standard deviation/slope of the calibration curve. The LODs were in the range of 0.09-0.18 µg/mL and LOQs were between 0.28-0.54 µg/mL. The analytical figures of merit for parabens were given in Table 1.

Precision was examined by intra- and interday studies at 0.50, 2.50 and 10.00 µg/mL. The standard

solutions at three concentration levels were analyzed triplicate consecutively in one day and triplicate in three different days (Table 2). The results were calculated as percentage of relative standard deviation (RSD%). The accuracy was determined as the recovery percent (%) (Table 2). All of the RSD% values were lower than 2.03 and the recoveries were higher than 96%.

Optimization of USVADLLME procedure

To optimize the USVADLLME procedure extraction solvent type, extraction solvent volume, disperser solvent volume, NaCl concentration, ultrasonication and vortex times were examined. All of the trials were performed in triplicate. The extraction recovery values were evaluated to determine the optimum extraction conditions.

Optimization of the extraction solvent type and volume

A literature survey revealed that chlorinated organic solvents were effective for the extraction of parabens from various sample matrices.[13,14] Considering that the extraction capabilities of CH_2Cl_2 and $CHCl_3$ were examined. Selected solvents match the criteria for liquid-liquid extraction since they had higher density than the sample solutions, were poorly soluble in the sample solutions and volatile enough to be easily separated. The extraction trials were performed with the standard solution which contained each analytes at a concentration 0.02 µg/mL. $CHCl_3$ provided better extraction recoveries for all the analytes (Fig. 3). Different volumes of $CHCl_3$ as 100, 150 and 200 µL were used to determine the optimum extraction solvent volume and 150 µL provided almost the same extraction performance with 200 µL (Fig. 4).

Optimization of the disperser solvent volume

A cloudy solution of the sample and the extraction solvent is formed by the use of the disperser solvent which determines the degree of dispersion. The disperser solvent was MeOH because of its good dispersing property in mixtures of water and $CHCl_3$. The extraction trials were performed by 25, 50, 100

μL of MeOH and 50 μL of MeOH was suitable for a complete dispersion with similar recovery results obtained by higher volumes (Fig. 5).

Optimization of the NaCl concentration

The presence of NaCl lowers the solubility of the parabens in the aqueaous phase by the salting-out effect. Trials were performed by no addition of NaCl and addition of NaCl at concentrations of 2 g NaCl/Land 3 g NaCl/L. The optimum concentration was 2 g NaCl/L (Fig. 6).

Optimization of the ultrasonication and vortex times

The time ranges of 30, 60 and 90 seconds were examined to determine the optimum ultrasonication time for high recovery of the parabens, whereas the effect vortex time was examined at 2, 4 and 6 minutes. Recoveries higher than 80.00% were achieved with an ultrasonication time of 90 seconds and vortex time of 4 minutes (Fig. 7 and Fig. 8).

Real sample analysis

The developed procedure was used for the extraction and determination of six parabens simultaneously in a cosmetic tonic (T), a micellar water (MW) and a synthetic urine sample. The conditions were optimized as: extraction solvent volume, 150 μL; dispersing solvent volume, 50 μL, NaCl concentration, 2 g/L; ultrasonication time, 90 sec, vortex time, 4 min. Since the samples were paraben-free, they were spiked at 0.02 μg/mL prior to the extraction procedure. The extraction procedure and the HPLC-FD analyses were performed triplicate. Recoveries were higher than 82% and the enrichment factors were in the range of 41.07-47.77 (Table 3).

The optimized USVADLLME procedure provided determination of parabens in different matrices at 20 ng/mL. Yılmaz et al. (2020) also developed an HPLC-FD method for the determination of different parabens (MP, EP, PP, IBP and BzP) in cosmetics.[29] Distinctly, in that study a pre-concentration method

was not applied. The LOQs were in the range of 0.88-0.97 μg/mL and it was not possible to quantify analytes at lower concentrations. The present procedure can be used for much lower concentrations with good precision and accuracy, which is an important advantage especially for biological samples. In addition, USVADLLME may be effective on the separation of various interferences in complex matrices. On the other hand, the sample preparation time is longer and a chlorinated solvent as chloroform is used for the extraction. However, LOQs are lower without requirement of a more sophisticated instrument like LC-MS or GC-MS.

Conclusions

To our best knowledge, the present report could be considered as the first research on the determination of the selected parabens simultaneously by USVADLLME-HPLC-FD. Reliable paraben analysis could be achived by the developed and validated HPLC-FD method. The proposed extraction procedure provided quantification of parabens at 20 ng/mL level without using a more sophisticated instrument like LC-MS or GC-MS, was easy to perform, and could be used for different aqueous personal care product and urine matrices. Also the use of low volumes of the extraction and dispersing solvents lowers the cost.

Acknowledgements

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Table 1. Analytical figures of merit for parabens

Analyte	t_R (min)[a]	Calibration range (µg/mL)	Linear equation	r	LOD (µg/mL)	LOQ (µg/mL)	Tailing factor (t)	Resolution (R_s)
MP	8.168±0.004	0.50-10.00	y = 1114911x - 105530	0.9972	0.10±0.01	0.30±0.02	1.250±0.009	-
EP	13.031±0.010	0.50-10.00	y = 1209791x - 320800	0.9956	0.10±0.02	0.30±0.03	1.293±0.010	8.459±0.013
IPP	20.660±0.013	0.50-10.00	y = 1205857x - 129613	0.9973	0.10±0.02	0.30±0.02	1.325±0.017	9.506±0.032
PP	23.229±0.017	0.50-10.00	y = 1134769x - 123760	0.9973	0.09±0.01	0.28±0.01	1.356±0.012	2.595±0.011
IBP	41.523±0.037	0.50-10.00	y = 1329085x	0.9970	0.12±0.03	0.36±0.03	1.273±0.017	13.343±0.099

BP	44.521±0.042	0.50-10.00	- 158082 y = 1248312x - 164808	0.9976	0.18±0.03	0.54±0.03	1.447±0.024	1.709±0.007

a. Mean ± SD, $n = 6$.

Table 2. Precision and accuracy of the developed HPLC-FD method

		MP	BP	IPP	PP	IBP	BP
Intraday	C[c] (µg/mL)						
($n = 3$) [a]	0.50	1.43	1.30	1.58	1.81	1.26	1.61
	2.50	0.61	0.52	0.56	0.50	0.62	0.74
	10.00	0.60	0.29	0.25	0.23	0.22	0.27
Interday	C (µg/mL)						
($n = 3$)	0.50	1.59	1.77	1.32	1.12	1.28	1.42
	2.50	1.82	1.65	1.66	1.46	1.42	1.45
	10.00	1.80	1.94	2.01	2.03	1.54	1.58
Recovery	C (µg/mL)						
(%)	0.50	98.41±1.77	96.71±1.18	101.34±1.15	101.11±1.26	100.74±1.40	99.67±1.67
($n = 3$) [b]	2.50	98.46±0.61	98.50±0.52	98.16±0.55	98.28±0.48	99.07±0.61	98.75±0.73
	10.00	98.55±0.59	98.61±0.29	98.68±0.25	98.76±0.22	99.78±0.22	99.94±0.27

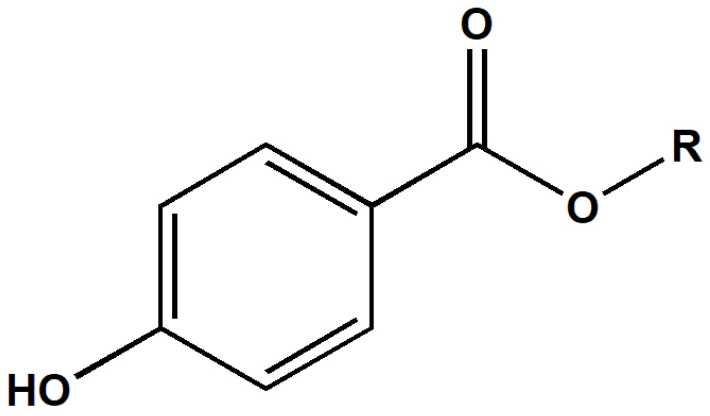
a. Relative standard deviation (%)
b. Recovery% ± standart deviation
c. Concentration (µg/mL)

Table 3. Analysis results of the spiked (0.02 µg/mL) real samples [a]

Sample	Analyte	Recovery (%)	RSD (%)	Enrichment factor
TS	MP	82.13±2.01	2.45	41.07
	EP	84.53±2.44	2.89	42.27
	IPP	88.53±3.03	3.42	44.27
	PP	92.80±2.88	3.11	46.40
	IBP	94.93±1.22	1.29	47.47
	BP	95.47±2.44	2.56	47.73
MW	MP	83.85±1.41	1.68	41.93

	EP	88.90±1.64	1.84	44.45
	IPP	90.52±0.55	0.61	45.26
	PP	94.99±1.37	1.44	47.50
	IBP	95.53±1.05	1.09	47.77
	BP	94.89±1.82	1.91	47.45
Urine	MP	95.03±2.01	2.45	47.52
	EP	94.51±2.44	1.89	47.26
	IPP	98.09±3.03	2.03	49.05
	PP	92.86±2.88	2.10	46.43
	IBP	94.96±1.22	1.85	47.48
	BP	95.45±2.44	2.01	47.73

a. $n = 3$



R: CH_3, methyl paraben
R: C_2H_5, ethyl paraben
R: C_3H_7, propyl and isopropyl paraben
R: C_4H_9, butyl and isobutyl paraben

Fig. 1 Chemical structures of the analyzed parabens.

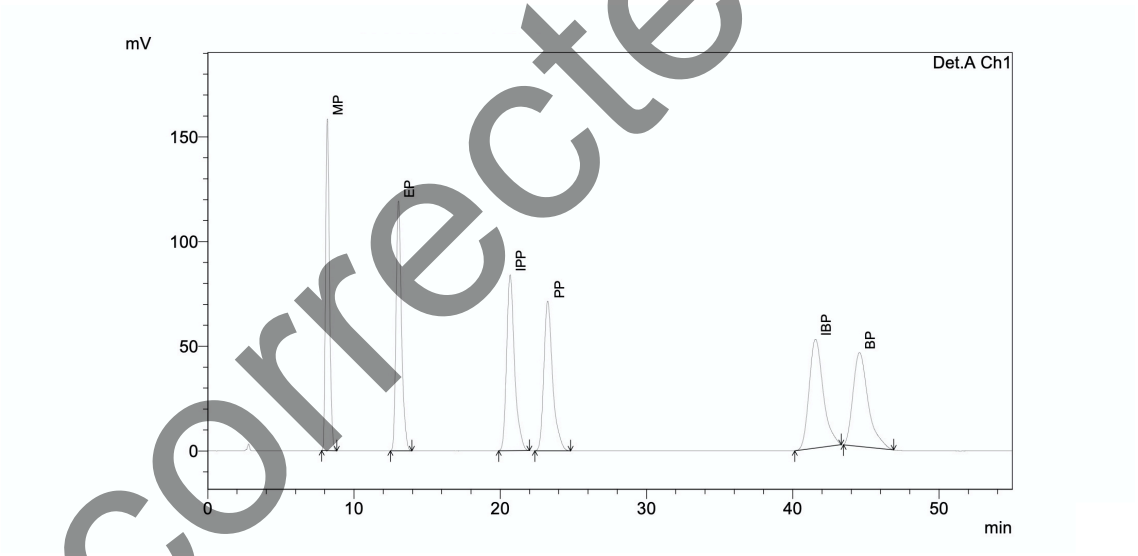


Fig. 2 A representative HPLC-FD chromatogram of the analyzed parabens (at 2.50 µg/mL).

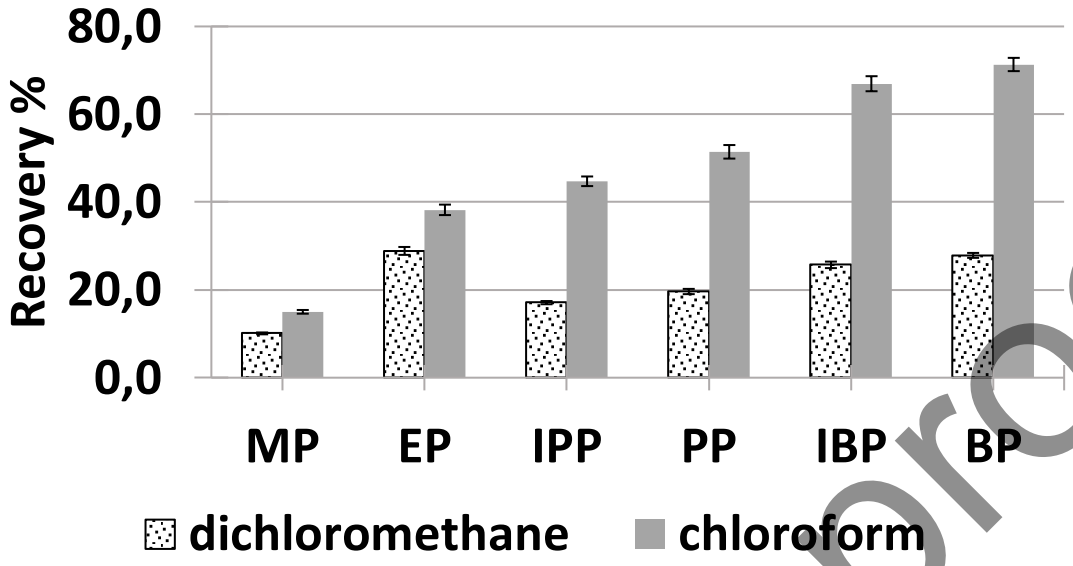


Fig. 3 Optimization of extraction solvent type. Disperser solvent (MeOH) volume, 50 µL; extraction solvent volume, 100 µL; ultrasonication time, 30 sec; vortex time: 4 min. n = 3, RSD values were in the range of 2.12-3.05%.

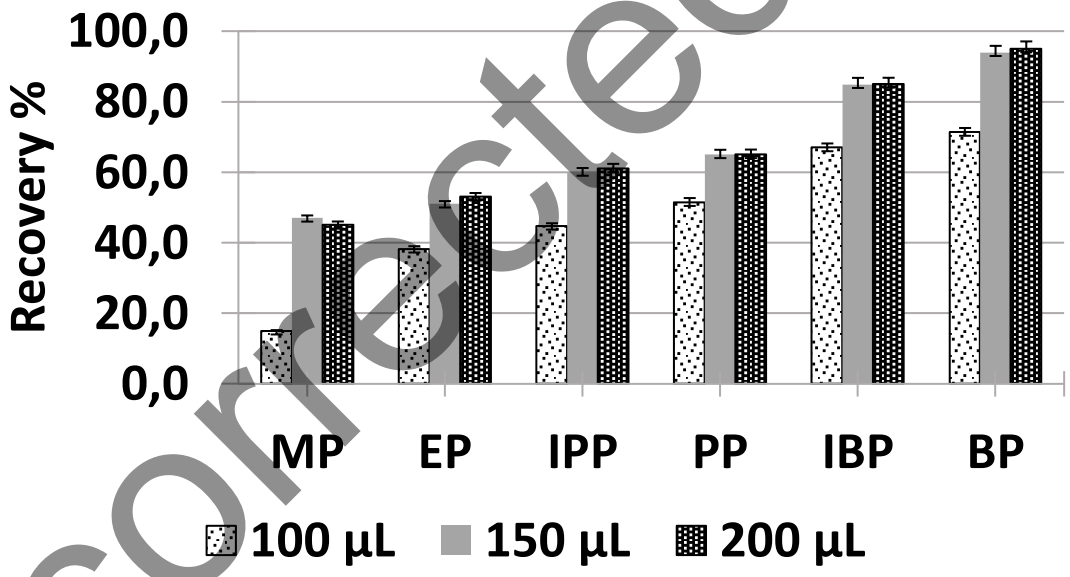


Fig. 4 Optimization of extraction solvent volume. Extraction solvent, CH_3Cl; disperser solvent (MeOH) volume, 50 µL; ultrasonication time, 30 sec; vortex time: 4 min. n = 3, RSD values were in the range of 1.59-2.25%.

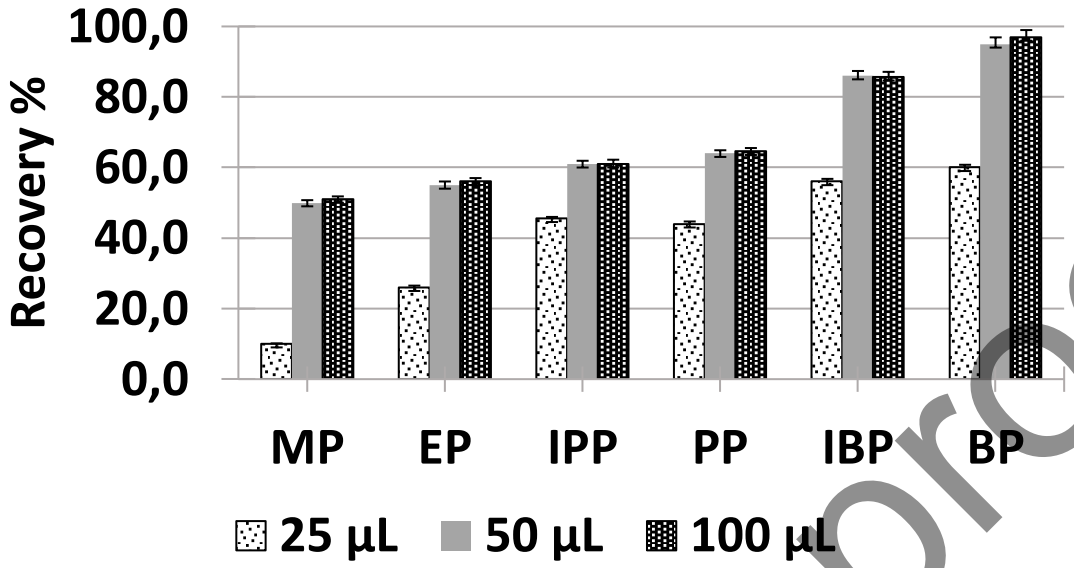


Fig. 5 Optimization of disperser solvent volume. Extraction solvent (CH_3Cl) volume, 150 µL; disperser solvent, MeOH; ultrasonication time, 30 sec; vortex time: 4 min. n = 3, RSD values were in the range of 1.00-2.10%.

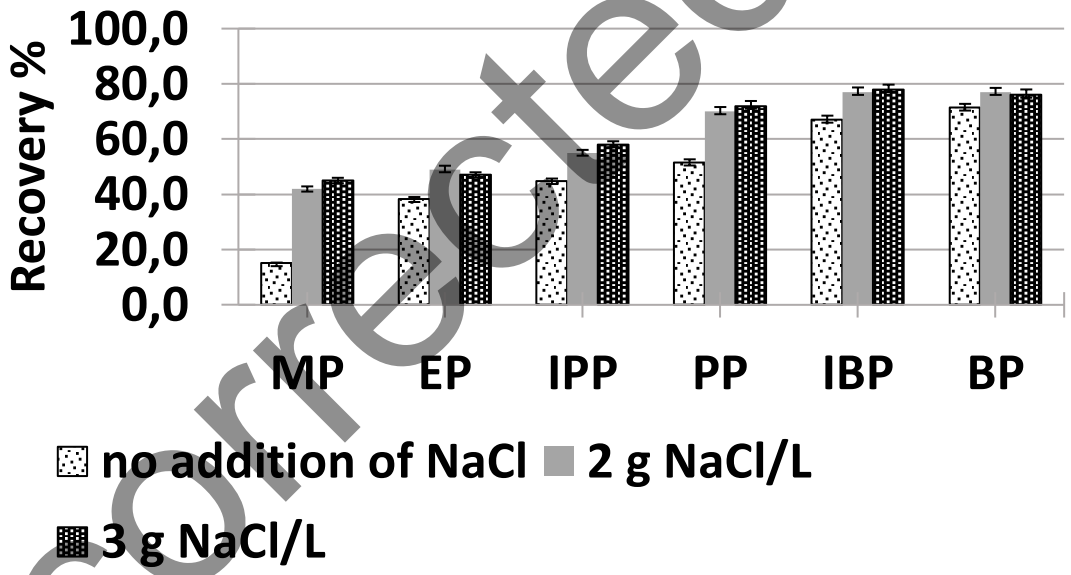


Fig. 6 Optimization of NaCl concentration. Extraction solvent (CH_3Cl) volume, 100 µL; disperser solvent (MeOH) volume, 50 µL; ultrasonication time, 30 sec; vortex time: 4 min. n = 3, RSD values were in the range of 2.08-2.79%.

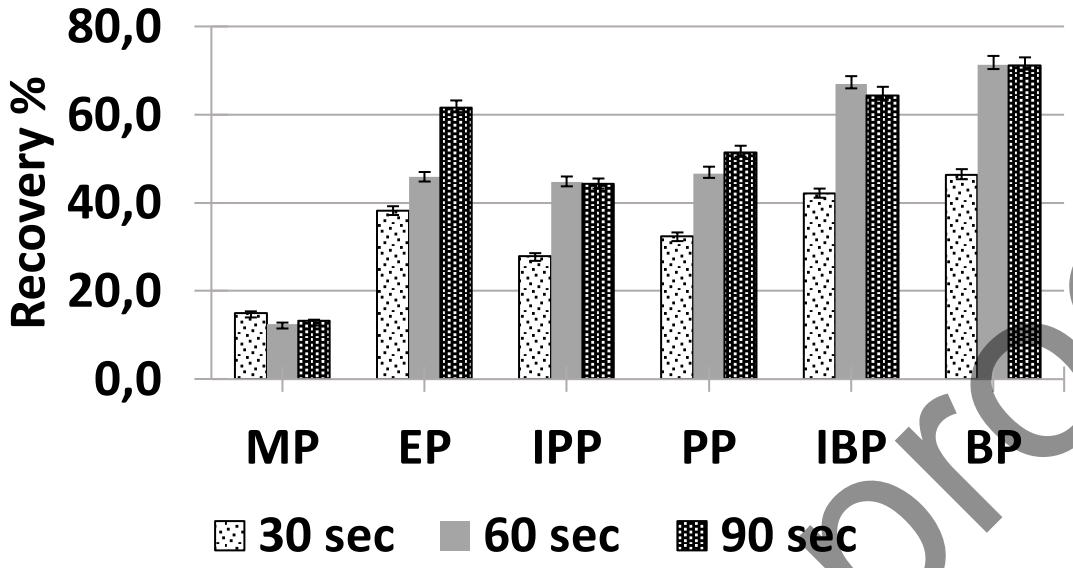


Fig. 7 Optimization of ultrasonication time. Extraction solvent (CH_3Cl) volume, 100 µL; disperser solvent (MeOH) volume, 50 µL; vortex time: 4 min. *n* = 3, RSD values were in the range of 2.51-3.12%.

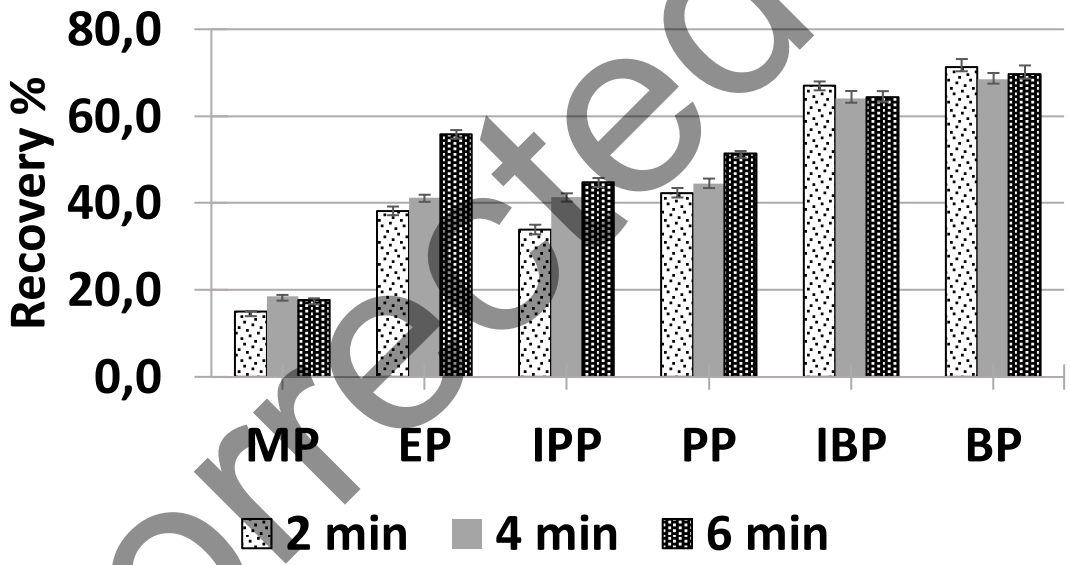


Fig. 8 Optimization of vortex time. Extraction solvent (CH_3Cl) volume, 100 µL; disperser solvent (MeOH) volume, 50 µL; ultrasonication time: 30 sec. n = 3, RSD values were in the range of 1.15-2.93%.