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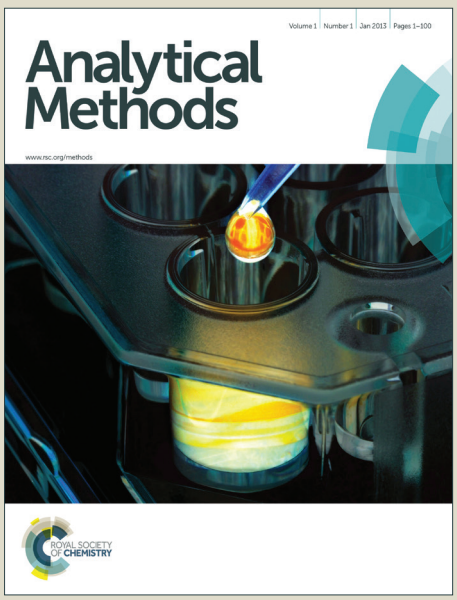

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Development of sensitive and rapid liquid-phase microextraction method followed by liquid chromatography mass spectrometry for the determination of zearalenone residues in beer samples

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Abstract

In this work, a new, rapid and sensitive, dispersive suspended microextraction procedure, followed by liquid chromatography mass spectrometry, was employed for the determination of zearalenone in beer samples. The liquid-phase microextraction method is based on solvent dispersion and magnetic stirring. Validation was carried out according to the Commission Decision 2006/406/EC guidelines. Under the optimum conditions, limit of detection (LOD) was determined at 0.44 μg kg^{-1} and the limit of quantification was calculated at 1.45 μg kg^{-1}. The relative standard deviation (RSD, n =5) for inter-day precision was found to be 5.4 % and for intra-day precision 3.6 %. The linearity was obtained by five points in the concentration range of 1.5-45 μg kg^{-1} and the recovery was > 91%. The occurrence of the selected mycotoxin was studied in several beer samples collected from local market from the region of Epirus.

Keywords: Dispersive suspended microextraction; Mycotoxins; Zearalenone; Beers; LC-MS.

1. Introduction

Mycotoxins are toxic natural secondary metabolites produced under particular environmental conditions by molds on agricultural commodities such as maize, wheat, oats and barley, in the field or during storage.[1-6] The occurrence, amount and type of mycotoxin may depend on the environment, fungi species, infection severity and of the kind of crop.[7, 8] Food contaminated by mycotoxins could be potential hazards to consumers[9]and could produce acute and chronic effects.[10]

Zearalenone (ZON) belongs to type B of trichothecenes.[11] It is an estrogenic resorcyclic acid lactone compound mainly produced by Fusarium graminearum and is classified as group 3 carcinogen in 1993 and 1999 by IARC. [12, 13] The main concern is that ZON is a naturally occurring estrogen that is well recognized to cause hormonal and endocrine disrupting effects in animals.[14-18]

Since cereals are raw materials of beer and beer-based drinks, it is necessary to consider that the risk of ZON and other mycotoxins contamination exists in such products. Beer is the oldest alcoholic beverage that contains water, alcohol, protein substances, mineral salts, carbohydrates[19] and according to the World Health Organization, the European countries are consuming the largest quantity of beer. European Union has established a maximum permitted limit for ZON at 100 μg kg^{-1} for cereals and suggested 20 μg kg^{-1} for their sub products.

To deal with the increasing number of sample matrices and mycotoxins of interest, fast and accurate analytical methods are needed for their determination in different commodities. Numerous analytical methods have been developed for the determination of mycotoxins in beer samples. [20-30] In recent years, there is a shift to the development of new liquid-phase microextraction protocols that make analysis faster and cheaper by using smaller volumes of solvents. These methods include

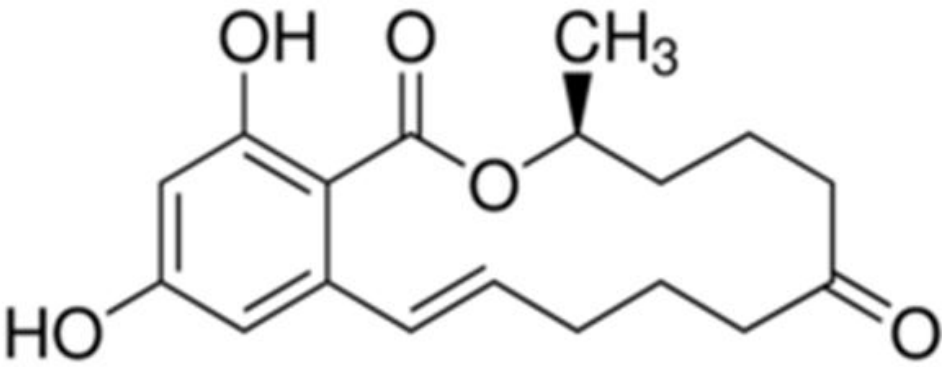
dispersive liquid-liquid microextraction based on solidification of floating organic droplet (DLLME-SFO), [31-33] temperature-controlled ionic liquid dispersive liquid–liquid microextraction(TC-IL-DLLME),[34] manual shaking-enhanced ultrasound-assisted emulsification microextraction (MS-USAEME),[35,36] dispersive liquid–liquid microextraction (DLLME),[37] hollow fiber-based liquid phase microextraction (HF-LPME),[38] array capillary in-tube solid-phase microextraction (ACIT-SPME),[39] single drop microextraction (SDME)[40] and dispersive suspended microextraction (DSME)[41]. With the above in mind, the aim of the present study was to develop a liquid-phase microextraction method based on DSME followed by liquid chromatography for the determination of ZON in beer samples. The novelty of the method relies not only in the introduction of a new extraction method that is employed for the first time in such matrix, but also to its efficiency to eliminate the bottleneck of LC/MS analysis in food samples, co-eluting matrix interferences, by the combination of a dilution factor of 1 to 5 and external matrix-matched calibration.

2. Experimental

2.1. Chemicals and reagents

Zearalenone ≥98% (ZON, Scheme 1) analytical standard was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). The individual stock solution of ZON at concentration of 100 mg kg^{-1} was prepared in acetonitrile, purchased from Panreac (Barcelona, Spain), and kept at -20 ^{o}C, in the dark. Before use, the solution was brought to room temperature. Working standard solutions were prepared using adequate aliquots of standard stock solution and diluting in acetonitrile. Analytical LC grade solvents, acetonitrile and water, were obtained from Fischer Chemicals (Leics,

UK). Toluene, hexane, isooctane, and p-xylene were supplied by Labscan (Dublin, Ireland). Potassium dihydrogen phosphate (KH_2PO_4), which was used for the preparation of buffer solution, was purchased from Merck (Darmstadt, Germany).



Scheme 1. ZON chemical structure

2.2. Apparatus

The analysis was carried out using liquid chromatography (LC-MS Schimadzu, Japan), equipped with an auto sampler (SIL-20A, Schimadzu, Japan) and a mass spectrometry detector (LC-MS 2010- EV, Schimadzu, Japan). The analytical column was a Pinnacle II C18 (150 x 4.6 mm, 5µm Restek, Bellefonte, PA). Positive and negative ionization mode using an isocratic gradient consisted of methanol:acetonitrile (60:40% v/v) - based on earlier investigations with ZON[42] - was evaluated for the detection of the target analyte in beer samples. During LC-MS ESI (-) mode the resulting spectra are simpler with fewer background ions compared to LC-MS ESI (+), since negative ionization essentially produces only the deprotonated $[M-H]^-$ ion (m/z=317.1), while at the same time the signal-to-noise ratio is

significantly improved. Concerning the ionization mechanism, it has been stated that in some circumstances neutral analytes can be converted to negatively charged species simply by loss of a proton as they pass through the sprayer capillary[29]. Mass spectrometry was used in selected ion monitoring (SIM) mode, with the following selected ions m/z: 317.1 and 318.1. The nebulizer pressure was 75 bar (1125 psi), the detector voltage (V) 1500 and flow rate 0.5 mL min^{-1}. Under these chromatographic conditions the retention time of ZON was 3.7 min.

2.3. Sample Collection and preparation

Commercial available domestic and imported bottled beers were collected from different stores, from the region of Epirus, and kept at 4 ^{o}C, in a dark and dry place. Each bottled beer sample was gently shaken and approximately 1 mL was degassed by ultra-sonication (Elma-S 30 H, Made in Germany) and diluted 1 to 5 (total volume 6.0 mL) with buffer solution adjusted to pH 5. The samples with undetectable levels of ZON (blank samples) were used for optimization and validation studies.

2.4. Dispersive suspended microextraction (DSME) procedure

When needed, the degassed and diluted (1:5) beer sample was fortified with a solution of the target compound to obtain the desired final concentration. A 550 μL aliquot of toluene was delivered, as extraction solvent, to the surface of the sample. A magnetic micro stirring bar (10 mm × 3 mm o.d., VWR, Arlington Heights, Illinois, USA) was placed at the bottom of the vial and the latter was placed on the magnetic stirrer (Hotplate Stirrer, LMS-100, Daihan Labtech, Made in Korea).

The DSME procedure first involved an extraction step, followed by a restoration step. In the extraction step (from opening the magnetic stirrer at a higher rate, to reducing its speed to the final restoration speed), the mixture was agitated for 30s (extraction time) at 800 rpm (extraction speed) and formed a cloudy solution (aqueous sample and toluene). ZON was extracted into the fine toluene droplets.

When the stirring speed was adjusted to 400 rpm, the restoration step began. At this stirring speed, a gentle vortex was formed and the toluene droplets began to coalesce. After 14 min (restoration time), the organic phase had absolutely separated from aqueous phase and had formed the final suspended phase in the bottom center of the vortex (500 μL). Then, the suspended phase was withdrawn and 20.0 μL of the organic phase was injected into the LC-MS for further analysis.

3. Results and discussion

3.1. Preliminary studies

Concerning the DSME extraction method, several factors may affect its efficiency, such as, extraction solvent, extraction solvent volume, and extraction time. During method development, the first consideration was to select an appropriate extraction solvent to recover efficiently the study compound. The solvent should fulfill the following criteria: i) to be insoluble in water in order to form two different phases in solution and facilitate the phase separation, ii) to exhibit a lower density than water to rise above the aqueous phase, iii) to have a high extraction capability toward ZON and iv) to display good liquid chromatographic behavior considering the matrix complexity. The solvents studied were toluene, hexane, isooctane, *p* xylene and

benzene. The results revealed that toluene had the highest extraction efficiency (75%) compared to the other tested solvents (hexane 60%, isooctane 64%, p-xylene 69%, benzene 72%).

Matrix effects are common problems that occur when using LC/MS and have adverse impacts on the analytical results[28]. More specifically, electrospray ionization is susceptible to matrix-related signal suppression/enhancement that is attributed to the competition between the analyte ions and matrix components[20]. To evaluate the presence and extension of this effect, ZON standards of different concentrations were analyzed in pure solvent and beer matrix. The combination of a dilution factor of 1 to 5 (beer to water), as well as, the use of matrix-matched standards (according to Council Decision 2002/657/EC (European Community 2002) and document SANCO/10684 (2009)) compensated the suppression signal effects, achieving an accurate quantification (matrix effect less than 18% was calculated and was considered as no matrix effect, because this variation would be close to the repeatability values) (Figure S1). Our observation was in agreement with Rubert et al.[25] who showed that the addition of established internal standards did not significant compensate the matrix effect compared to external matrix-matched calibration. In light of these results, the choice of toluene as extraction solvent, as well as, of the selection of dilution factor 1:5, were done with respect to resolve and diminish effects from the matrix constituents and make it applicable in real samples with a minimum of sample preparation (Fig. 1).

<Fig. 1>

Moreover, some "technical" details of the DSME procedure were preliminary evaluated before optimization. The extraction speed was adjusted to 800 rpm due to the adequate dispersion of toluene to diluted beer sample and to the easiness of retrieving the droplets. The adjusted restoration speed of 400 rpm forced the droplets to gather up at the top center position of the vortex. When a restoration speed higher than 400 rpm was selected, the suspended phase was not stable and it was hard for the dispersive droplets to gather up. On the contract, when the restoration speed was below 400 rpm, a vortex was not created at the center of the vial, so it was hard to withdraw the droplets with the micro-syringe. Finally, the restoration time of toluene droplet was calculated to be 14 min, until all the dispersive droplets were gathering up.

3.2. Optimization of extraction time

In the DSME, extraction time is defined as the time from opening the magnetic stirrer to turning down to the restoration speed. The extraction of ZON from the diluted beer samples into the organic solvent is an equilibrium process. The effect of extraction time was evaluated over the time range between 10s and 60s. Time shorter than 30s does not permit adequate dispersion of toluene to the entire mass of the sample, so the extraction yield of ZON was low (Fig. 2). Increasing from 40s to 60s, the reunification of the droplets became very difficult and it was impossible to carry out further analysis. This phenomenon revealed that the contact surface between organic phase and aqueous phase was infinitely large and the equilibrium state was obtained in a few seconds. Based on the above considerations, 30s was selected as the extraction time.

<Fig. 2>

3.3. Optimization of extraction solvent volume

In order to achieve the best extraction efficiency, different volumes of toluene (100 - 600 μL) were examined. As well, the diluted beer sample was settled to 6.0 mL. Results have shown that addition of toluene at the range of 100 – 200 μL, is not efficient for ZON extraction, due to toluene solubility to ethanol, that resulted at a small recovered/restored solvent phase, which was difficult to withdraw. At higher toluene volumes (250 to 500 μL, Fig. 3) the extraction of the target analyte to the organic solvent was increased and 550 μL was selected as the most adequate, since under these conditions, toluene was more efficiently dispersed in the diluted beer sample and restored to form the organic droplet. At higher toluene amounts there was not any notable improvement or recrudescence of the extraction yield.

<Fig. 3>

3.4. Optimization of pH addition

Different pH values, ranging from 3 to 10 (including the pH of diluted beer sample), were studied in order to evaluate its influence in the DSME process. Results revealed that the best response for ZON was accomplished at pH 5 (Fig. 4), which was selected

for further experiments. The environmentally relevant p*Ka* value of ZON is defined to 7.6. While at *pH* >9.6 the anion is dominant in the solution, at p*H*<5.6 is mainly in the neutral form, and therefore ZON was efficiently extracted from toluene.

<Fig. 4>

3.5. Optimization of ionic strength

Addition of salt to the sample may have several effects on the DSME. Ionic presence on the extraction procedure, was investigated with sodium chloride concentration in the range of 0-6% (w/v).The effect of salt concentration on the extraction efficiency of ZON is illustrated in Fig. 5. As can be seen, the addition of salt 3% w/v has a positive effect on the extraction efficiency of the target analyte. Salt attracts water molecules in an effort to "solvate" the ions therefore decreasing the solubility of ZON and increasing the extraction yield. However, extraction efficiency for ZON decreases with the increase of NaCl concentration at higher values (6% w/v). This observation could be attributed to the formation of a physical barrier across aqueous phase and organic phase interface, which prevents the mass transfer of the target analyte into the extraction solvent[42]. Based on the above experimental results, ionic strength of 3% w/v (NaCl) was selected.

<Fig. 5>

3.6. Analytical performance of DSME

Performance characteristics of the optimized method were established by a validation procedure, studying trueness, precision, linear range, sensitivity and specificity.

3.6.1. Method trueness

Method trueness was assessed through relative recovery studies, spiking blank samples at two fortification levels (1.5 and 10.0 μg kg^{-1}) processing five samples in each experiment. The relative recovery was defined as the ratios of the peak areas of the analyses in the spiked blank samples and the peak area of the analyses in distilled water sample, spiked with the same amount of the analyte. Mean relative recovery calculated was 91% and were within the criteria approved by the European Committee for Standardization, for the acceptability of analytical methods (Commission Directive 2006/401/EC).

3.6.2. Precision

The intra-day repeatability (RSDr%, n=5) and inter-day reproducibility (RSD_R%, n=5 × 3 days) were expressed by calculating the relative standard deviation (RSD %). Experiments were carried out by extracting a beer sample spiked at 1.5 and 10 μg kg^{-1}. Intra-day repeatability and inter-day reproducibility were lower than 10%.

3.6.3. Linear range

Method linearity was evaluated by performing matrix-matched calibration curves at a concentration range of 1.5 to 45 μg kg^{-1}. The obtained peak area versus concentration was linear (least-squares linear regression) in the assayed range achieving coefficient of determination equal to 0.9974.

3.6.4. Limit of detection (LOD) and quantification (LOQ) and specificity

LODs and LOQs were calculated, analyzing blank samples spiked at 0.025, 0.050, 0.10, 0.50, and 1.0 μg kg^{-1}, and they were determined as the lowest concentrations of analyte for which signal-to-noise ratios were 3 and 10, respectively. The detection and quantification limits of ZON with the proposed method were 0.44 and 1.45 μg kg^{-1}, respectively. These values were below the guidance levels established by European Commission regulation (EU) for cereal sub-products (20 μg kg^{-1}).

The specificity of the method was tested by the analysis of blank samples. The absence of any chromatographic peak in every matrix, at the same retention time as ZON, indicated that there were no matrix compounds that might give a false positive signal in these blank samples (Fig. 1).

The developed DSME method could be equally compared in relation to the pretreatment time, sensitivity and LODs with other liquid-phase microextraction methods such as DLLME [28]. Recovery of ZON after DLLME and high performance thin layer chromatography (HPTLC) equipped with a densitometer was 83% while the proposed method provides up to 91%. The limits of detection were

quite comparable at 0.12 μg kg^{-1} (for DLLME) and 0.44 μg kg^{-1} (for DSME). However, limitation of both methods could be the use of toxic extraction solvents.

3.7. Application to real samples

The optimized method was applied to analyze seven blond beer samples collected from local stores from the region of Epirus. The results are depicted at Table 1. No ZON residues were found in analyzed beer samples. To validate the results, real samples spiked with known amount of ZON were analyzed. Good recoveries were obtained in the range of 86 to 95%.

[Table 1]

4. Conclusions

The present study reports a new, rapid, sensitive and low cost analytical method for the determination and quantification of a well-known endocrine disruptor, ZON, in the oldest and most worldwide consumed beverage. The extraction procedure allows the preconcentration of ZON by a factor of 2, while at the same time overcomes the problem of possible interfering compounds allowing a LOQ below the guidance levels established by European Commission regulation. The validated DSME was found to be suitable for the routine monitoring of ZON in beer samples by regulatory laboratories. Moreover, implementing the negative ion mode seems to be of general

applicability also to other mycotoxins and their metabolites. In such case, further studies should be carried out improving the chromatographic conditions and the efficiency of separation between the target analytes.

Acknowledgements

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Table 1 Analysis of different type commercial beers by the DSME method using LC-MS (n=3)

Sample	Added (μg kg^{-1})	Found (μg kg^{-})	Recovery (%)
Pilsner 1	–	N.D	N.D
	20.0	17.4 ± 0.2	87%
Pilsner 2	–	N.D	N.D
	20.0	18.4 ± 0.2	92%
Pilsner 3	–	N.D	N.D
	20.0	17.8 ± 0.3	89%
Lager 1	–	N.D	N.D
	20.0	18.2 ± 0.3	91.2%
Lager 2	–	N.D	N.D
	20.0	17.2 ± 0.1	86%
Ale 1	–	N.D	N.D
	20.0	18.8 ± 0.2	94%
Ale 2	–	N.D	N.D
	20.0	17.6 ± 0.2	88%

Legends of figures

Fig. 1 LC–MS chromatograms of a) direct analysis of degassed beer sample, b) analysis of degassed beer sample after dilution (1:5), c) analysis of degassed blank beer sample after dilution (1:5) and subjected to DSME, d) analysis of degassed beer sample after dilution (1:5), and subjected to DSME (spiked with 10 μg kg^{-1} ZON)

Fig. 2 Effect of extraction time on the efficiency of DSME

Fig. 3 Comparison of the extraction recoveries attained with different toluene volume

Fig. 4 Effect of pH on the extraction of ZON

Fig. 5 Extraction yields (%) of three different concentrations of salinity

Fig. 1

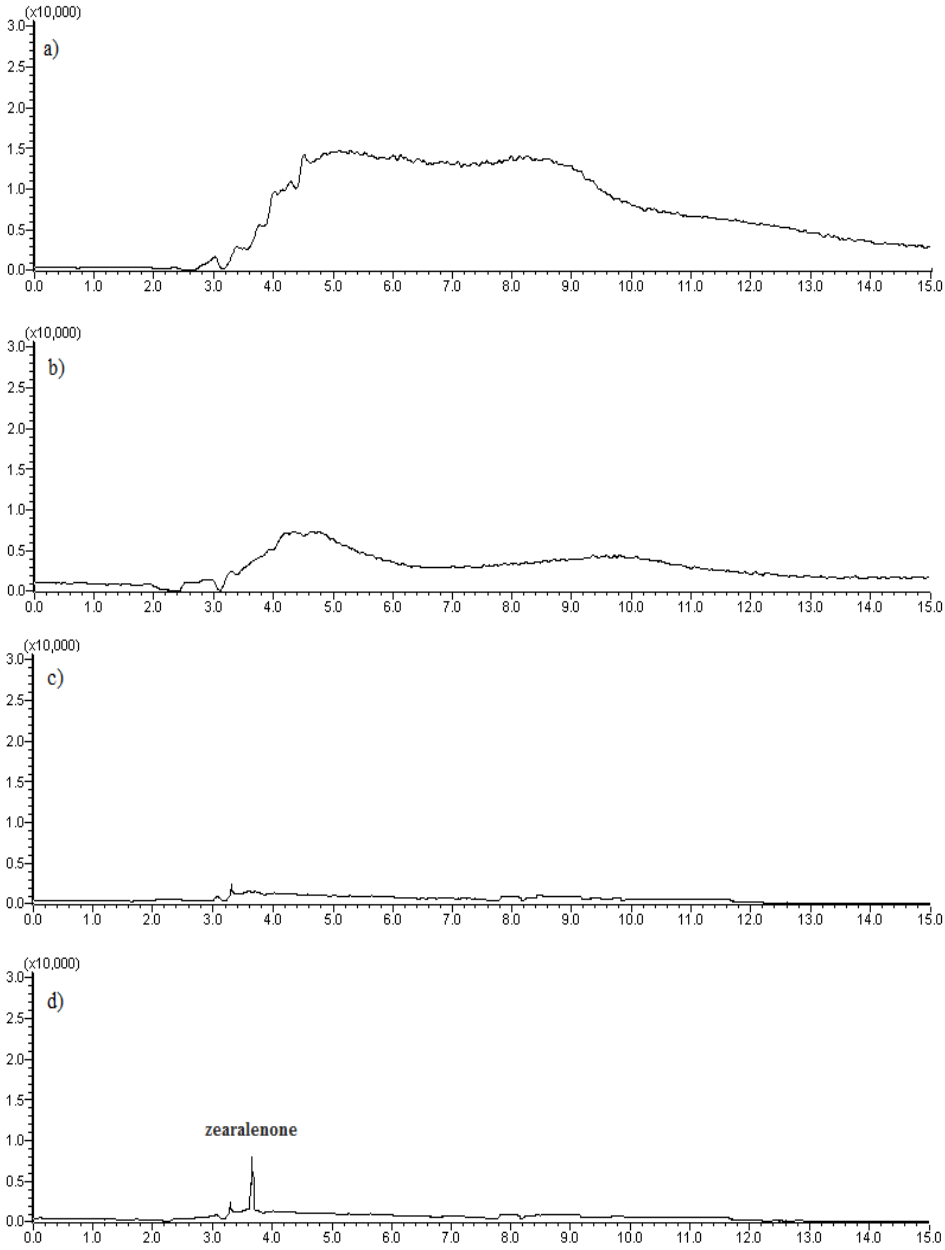

Fig. 2

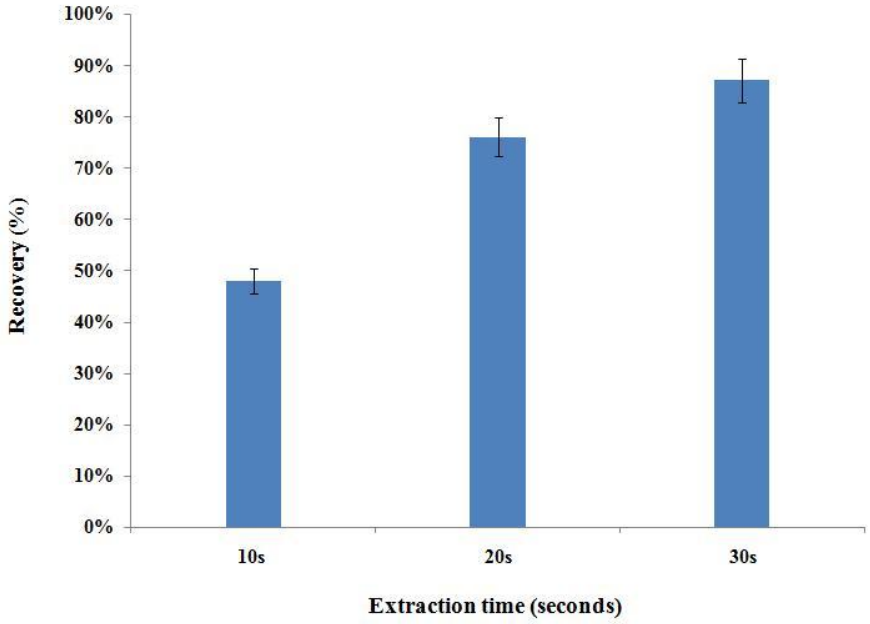

Fig. 3

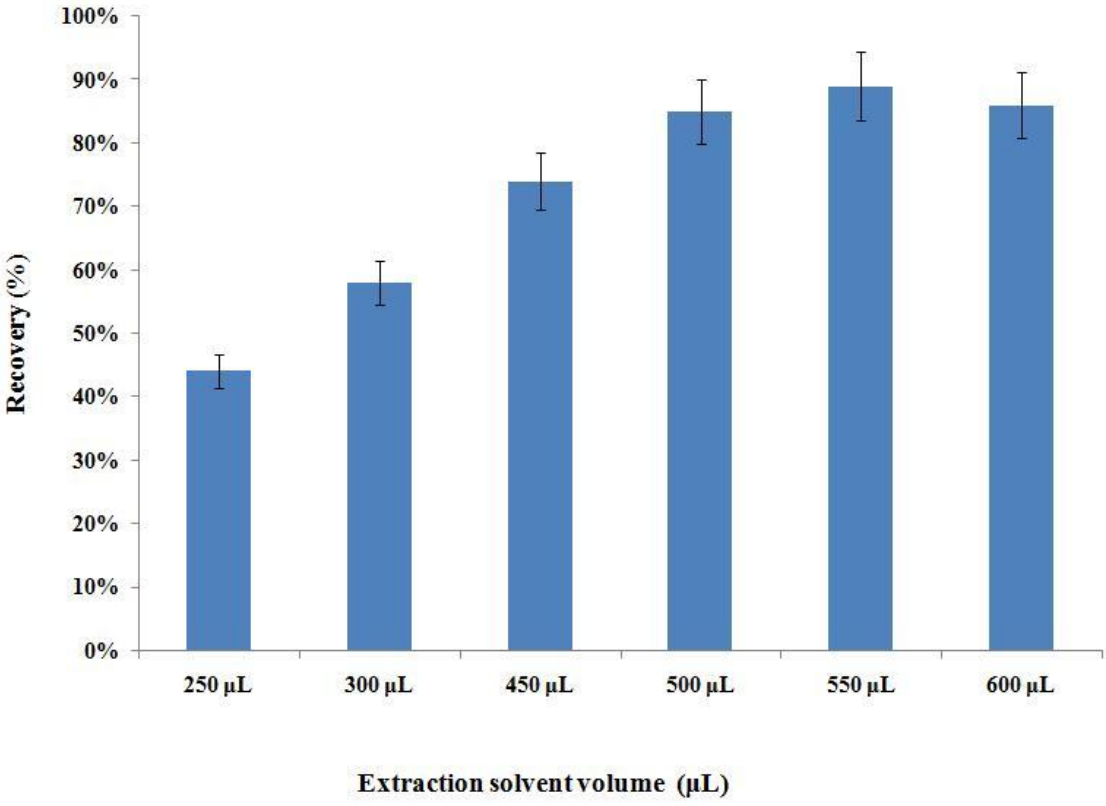

Fig. 4

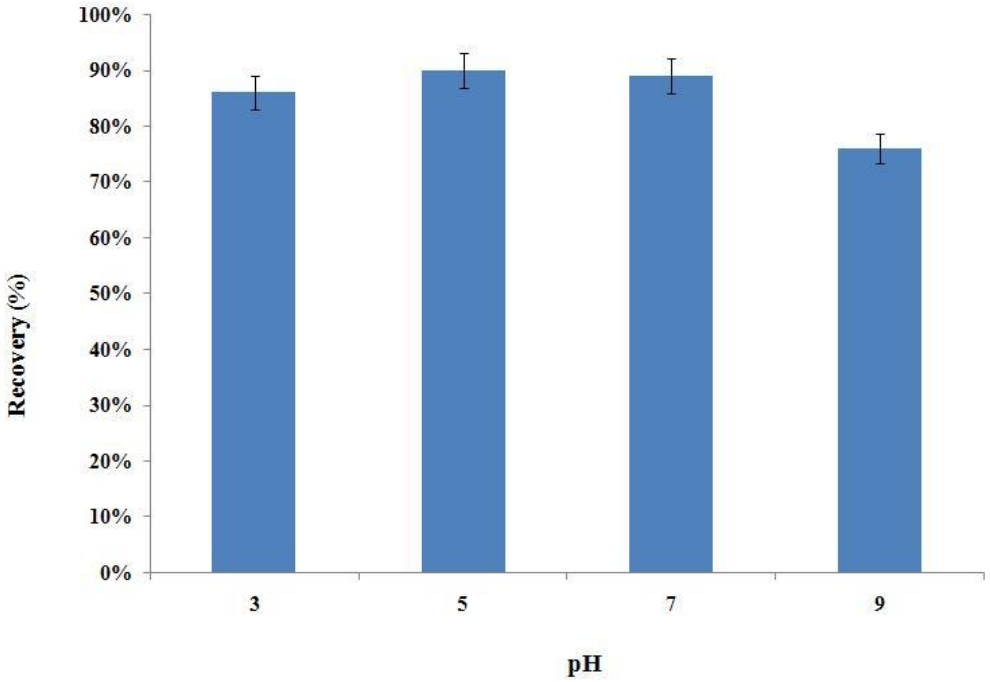

Fig. 5