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Salting-out Assisted Liquid-Liquid Extraction for the Determination of Multiresidue Pesticides in Alcoholic Beverages by High Performance Liquid Chromatography

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Abstract: A salting-out assisted liquid-liquid extraction (SALLE) method followed by high-performance liquid chromatography with ultraviolet-visible detector (HPLC-UV/Vis) has been proposed for determination of five multiclass pesticides residues including, atrazine, ametryn, terbutryn, carbaryl and chlorothalonil from various alcoholic beverages: beer, wine and Ethiopian honey wine (*Tej*). Experimental parameters influencing the extraction efficiency of the method such as the type and concentration of salt, volume of acetonitrile (the extraction solvent) and pH of the sample were assayed and the optimum conditions were established. Under the optimum experimental conditions, matrix-matched calibration curves were constructed using beer sample as the representative matrix and good linearity over wide concentration ranges were obtained with coefficient of determination (r^2) of 0.997 or better. The limits of detection (LOD) and quantification (LOQ) of the method, which were determined as 3 and 10 times a signal-to-noise ratio (S/N), were in the ranges of 1.3-3.9 and 4.5–12.8 μg L^{-1}, respectively. Precisions studied in terms of repeatability and intermediate precision (with-in lab reproducibility) at two concentration levels have demonstrated acceptable %RSD values, which were less than 10% in both cases. The applicability of the method was also investigated by analyzing various alcoholic beverages and demonstrated satisfactory recoveries in the ranges of 71-104% with their corresponding %RSDs less than 10% in all cases. The results of the study revealed that the developed SALLE method is selective and efficient sample preparation procedure prior to quantitative analysis of the target analytes by HPLC-UV/Vis.

Keywords: Salting-Out Liquid–Liquid Extraction, Multiclass Pesticides, Alcoholic Beverages, High Performance Liquid Chromatography, Ultraviolet-Visible Detector

1. Introduction

These days, various pesticides including herbicides, insecticides and fungicides are used to control and/or destroy pests that may affect crop yield. Nevertheless, their wide use in or on agricultural products might be resulted in the occurrence of residues of these chemicals and their metabolites in raw as well as processed food commodities, water and soil [1]. Alcoholic beverages which are usually prepared from various agricultural products and are considered as a part of human diet [2] could also be directly or indirectly contaminated by these pesticides and subsequently, could affect the health of consumers [3-9]. To monitor and control the health of consumers, legislative authorities and monitoring organizations of different countries, have set maximum residue limits (MRLs) of pesticide residues in various raw and processed foods, including alcoholic beverages [9-12]. Therefore, determination of the residual levels of pesticides in various

alcoholic beverages is crucial to ensure their safety consumption. In this regard, it is imperative to develop simple, rapid, selective and environmentally benign analytical methods for precise determination of trace level multiclass pesticide residues in different alcoholic beverages in order to monitor and control the use of pesticides.

Various analytical techniques have been reported for trace level determination of pesticide multiresidues in alcoholic beverages. Gas chromatography (GC) with various detectors including flame ionization detector (FID) [13, 14], electron capture (ECD) [15], nitrogen–phosphorus detection (NPD) [15, 16], mass spectrometric detector (MS) [7, 8, 17], tandem mass spectrometry (MS/MS) [5], capillary liquid chromatography coupled with diode array detection (cHPLC-DAD) [3], ultra-high pressure liquid chromatography coupled to MS/MS (UHPLC-MS/MS) [4] and liquid chromatography with MS/MS (LC-MS/MS) [18] have been used for determination of pesticides residues in wine. UHPLC-MS/MS [4], GC-ECD, GC-MS [19] and LC-MS/MS [20] were reported for determination of pesticide residues in beer [20]. HPLC-DAD [9] was reported for determination of pesticide residues in Ethiopian honey wine, locally called *Tej*, which is one of the national known home brewed Ethiopian alcoholic beverages and it is prepared from fermented honey and special kind of hops called Gesho *(Rhamnus prinoides)* [2, 9, 21, 22]. LC-MS/MS [6] was also reported for analysis of pesticide residues in honey liqueur.

Various sample preparation methods were reported for the selective extraction and preconcentration of pesticides residues, prior to their quantitative determination. In spite of its numerous disadvantages, such as time consuming, labor intensiveness, use of large sample size and high volumes of expensive and hazardous organic solvents, solid-phase extraction (SPE) [7, 8, 17, 18] has been widely used for extraction and/or preconcentration of pesticide residues from alcoholic beverages. Solid-phase microextraction (SPME) [5] has also been reported for extraction of pesticides from wine. However, SPME is expensive, uses fragile and easily broken fibres, has limited lifetime and sometimes sample carry-over could be a problem [23]. Other sample preparation methods such as supported liquid membrane (SLM) [9], microporous membrane liquid–liquid extraction (MMLLE) [13, 14], hollow-fibre liquid phase microextraction (HF-LPME) [4], ultrasound-vortex-assisted dispersive liquid-liquid microextraction (USVA-DLLME) [16], dispersive liquid-liquid microextraction (DLLME) [6] and quick, easy, cheap, effective, rugged and safe (QuEChERS) [6] as well as dispersive solid-phase extraction (d-SPE) [17] have also been reported for analysis of pesticide multiresidues in alcoholic beverages. Though, these procedures are relatively simple, rapid, cheap and attractive alternative for sample preparation, they are still demanding special equipment, reagents and/or toxic organic solvents.

Recently, salting-out assisted liquid-liquid extraction (SALLE) has gained particular recognition for extraction and/or pre-concentration of different organic compounds. SALLE is a variation of solvent extraction that uses water-miscible organic solvents such as acetone, acetonitrile, ethylacetate and isopropanol [26, 30, 36]. In the procedure, addition of appropriate amount of salts such as magnesium sulfate ($MgSO_4$), ammonium sulfate ((NH_4)$_2SO_4$), sodium chloride (NaCl), calcium chloride ($CaCl_2$), potassium carbonate (K_2CO_3) and calcium sulfate ($CaSO_4$) reduces the mutual miscibility of the two liquids, i.e., the aqueous sample and water-miscible organic solvent, causing formation of a two-phase system with the simultaneous extraction of the target analytes into the organic phase [36]. The method is simple, fast, cheap and environmentally friendly—and its extract is also compatible with HPLC/LC, CE and/or GC instruments [36, 37]. SALLE has been applied for extraction of various organic compounds including α-dicarbonyl compounds in beer [24] and wines [25]; biogenic amines in fruit juices and alcoholic beverages [26]; N-nitrosamines in skin care cosmetics [27]; trimetazidine in rat plasma [28]; methoxetamine in rat tissues (rat brain, liver and lungs) [29]; sulfonamides from different matrices (tea, water, milk, honey, human urine, plasma and blood) [30, 31]; temozolomide from plasma [32]; vitamin K homologues in human plasma [33]; multi-mycotoxin biomarkers in pig urine [34]; betalactam antibiotics in infant dairy products [35]; benzimidazole fungicides [36]; sulfonylurea herbicides in water and banana juice samples [37]; triazole pesticides in water samples [38]; multiresidue pesticides in surface, ground and drinking water [39]; and chlorophenols in wine [40]. However, to date there is no report on the use of SALLE procedure in combination with HPLC-UV/Vis for analysis of multiclass pesticide residues in alcoholic beverages.

Therefore, in this work, the SALLE method combined with HPLC-UV has been proposed for selective extraction and preconcentration as well as quantitative determination of five multiclass pesticides; namely, carbaryl, atrazine, ametryn, terbutryn and chlorothalonil from alcoholic beverages including wine, beer and Ethiopian honey wine (*Tej*). Various parameters affecting the extraction efficiency of the technique were investigated so as to establish the optimum conditions for extensive future applications.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals and reagents used in this study were of analytical grade while the solvents were of HPLC grade. Acetonitrile from Ashland Industries Italia S.R.L. (Busnago, Lombardy, Italy) and ultrapure water, obtained after purification with double distiller A8000 Aquatron water still (Bibby Scientific Ltd, Staffordshire, United Kingdom) were used as a mobile phase. Sodium carbonate (Na_2CO_3) and $MgSO_4$ were purchased from Fisher Scientific Company L. C. C. (Pittsburgh, USA); NaCl, ((NH_4)$_2SO_4$ and hydrated sodium hydrogen phosphate ($NaH_2PO_4.2H_2O$) obtained from BDH Laboratory supplies (Poole, England) were used during the experimental studies.

Analytical pesticide standards of carbaryl and chlorothalonil were purchased from Sigma Aldrich (St. Louis, MO, USA), whereas atrazine, ametryn and terbutryn were obtained from Dr. Ehrenstorfer (Augsburg, Germany). The chemical structures, common names, abbreviations and the pk_a of the target pesticides are given in Figure 1. Stock standard solutions containing 1000 mg L^{-1} of each pesticide were prepared in acetonitrile and stored in dark at 4ºC.

Intermediate working solution containing 20 mg L^{-1} of each standard was also prepared in acetonitrile. Working standard solutions were prepared daily from a mixed intermediate standard solution by diluting with deionized water and then used for optimization of the parameters affecting the SALLE procedure as well as method validation. All solutions were stored under refrigeration below 4°C when not in use.
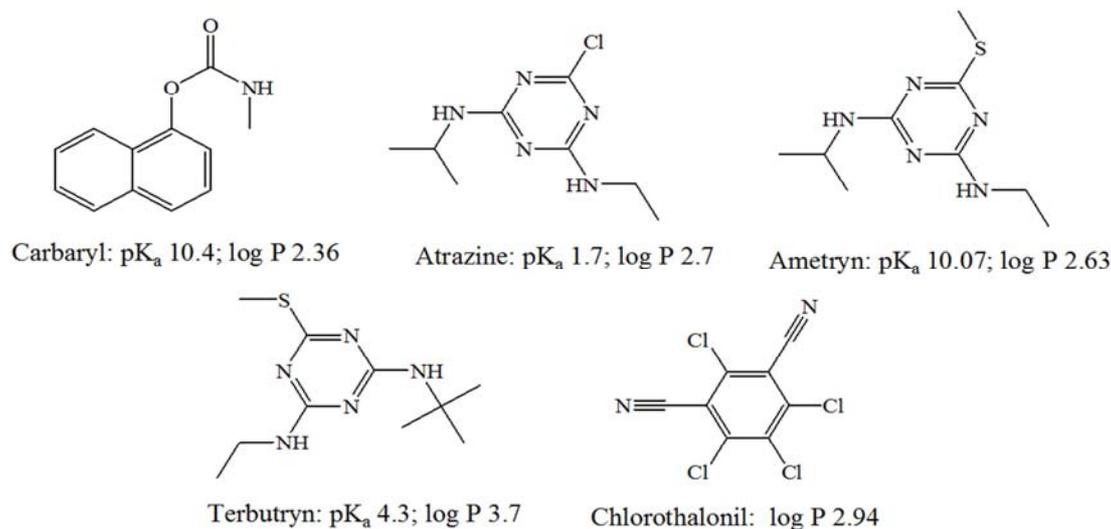

Figure 1. The chemical structures, common names, abbreviations and octanol-water partition coefficients (log P; at pH 7 and 20°C) and pk_a of the target pesticides.

2.2. Instruments and Equipment

Chromatographic analyses were performed using Agilent Technologies 1200 infinity series HPLC, equipped with quaternary pump, vacuum degasser, autosampler and UV/Vis detector all purchased from Agilent Technologies (Waldbronn, Germany). Chromatographic separation was carried out using Eclipse plus C_{18} column (100 x 4.6 mm I. D., 3.5 µm particle sizes) obtained from Agilent Technologies. Data acquisition and processing were accomplished with LC Chemstation software, (B.02, 01-R1).

A pH meter, Adwa, model 1020 (Romania, Europe) was used for measurement of pH. An ultrasonic heater, Dacon®, Dacon laboratories Ltd (St. Hove, East Sussex), centrifuge, Model 800 (China, Beijing) and 15-mL centrifuge tube, corning incorporated, (Corning, NY, Mexico) were used during sample preparation.

2.3. Chromatographic Conditions

Chromatographic separations were carried out based on the earlier reported findings [41]. An isocratic elution comprising of 45% ultrapure water (solvent A) and 55% acetonitrile (solvent B) was used throughout the analysis. Prior to the sample extract injection, the HPLC column was washed and conditioned with the mobile phase for 5 min. Analysis was performed at 0.5 mL min^{-1} flow rate, a column temperature of 30°C, 15 µL injection volume and a monitoring wavelength of 224 nm.

2.4. Alcoholic Beverage Samples

Three different kinds of alcoholic beverage samples were collected from local supermarkets in Addis Ababa, Ethiopia. Beer and red wine (Gouder red wine) samples which were manufactured by Meta Abo Brewery Share Company SC (Alem Gena, Sebeta, Ethiopia) and Awash Wine Share Company (Nefasilk Lafto sub-city, Addis Ababa, Ethiopia), respectively, were purchased from local market in Arada sub-city of Addis Ababa. Ehtipian honey wine "*Tej*" was purchased from Mekanisa area, Nefasilk Lafto sub-city of Addis Ababa. The collected samples were stored in the dark at 4°C until the analysis, without prior sample pretreatment.

2.5. SALLE Procedure

2.5 mL alcoholic beverage sample was taken into a 15-mL conical centrifuge tube. The sample was then spiked with appropriate concentrations of the target pesticides standards and kept for a minimum of 30 min to ensure homogeneity and attaining equilibration. Thereafter, 2.5 mL of 0.04 mol/L phosphate buffer (pH 7) and 1 mL acetonitrile were added, respectively and the content was then manually shaken for few seconds. Subsequently, 1.5 g of $MgSO_4$ was added and the mixture was again shaken for a minute, before subjecting to centrifugation for 5 min at 4000 rpm to enhance the phase separation. Eventually, 200 µL of the upper layer organic phase was carefully withdrawn using a micropipette and transferred into insert vial, which was housed in a 1.5 mL

autosampler vial and 15 μL of the extract was then injected into HPLC system for separation and quantification.

3. Results and Discussion

3.1. Optimization of SALLE Pprocedure

The SALLE method, usually involves optimization of various experimental variables that are potentially affecting the extraction efficiency and selectivity of the method [25, 30, 37]. These variables include the type and concentration of salt, volume of acetonitrile (the most commonly used extraction solvent in SALLE procedure) and pH of the aqueous samples. In this study, optimization of the parameters was carried out using 5 mL deionized water containing 200 μg L^{-1} standards of the five target analytes. The average peak area of the replicate analyses was used to evaluate the extraction efficiency of different experimental parameters.

3.1.1. Selection of Salt Type

In SALLE procedure, the degree of phase separation can be varied with the types of salts used [30, 31, 37]. In this study, the effect of four salts including NaCl, MgSO$_4$, Na$_2$CO$_3$ and (NH$_4$)$_2$SO$_4$ each at 20% *(m/V)* concentration level, were tested as potential salting-out agents. With the exception NaCl, the others have exhibited good phase separation. However, as can be seen from Figure 2, the highest peak areas of the analytes were observed when MgSO$_4$ was used as salting-out agent. This could be attributed to the highest ionic strength per unit concentration of MgSO$_4$ in aqueous solution as compared to the others [42]. Thus, MgSO$_4$ was selected for subsequent experiments.
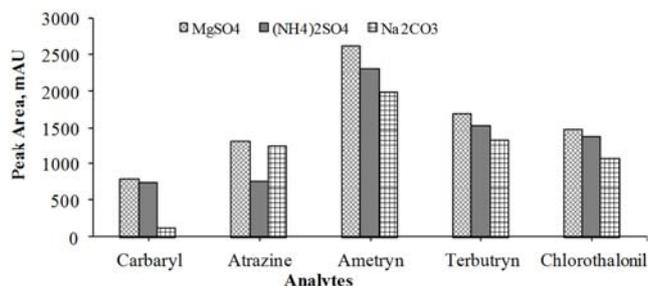

Figure 2. *Effect of salt type on SALLE efficiency. Experimental conditions: extraction solvent volume, 1 mL; centrifugation speed and time, 4000 rpm and 5 min, respectively.*

3.1.2. Effect of MgSO$_4$ Concentration

Concentration of the salt is also important factor affecting the extraction efficiency of the method [30, 37]. The concentration of the salt must be large enough to induce the required phase separation. Accordingly, different concentrations of MgSO$_4$ ranging from 10–40% (*m/v*) in the sample solution were assessed. It was observed that peak areas of the target analytes increase with concentration of the salt up to 30% and then decline at higher concentration

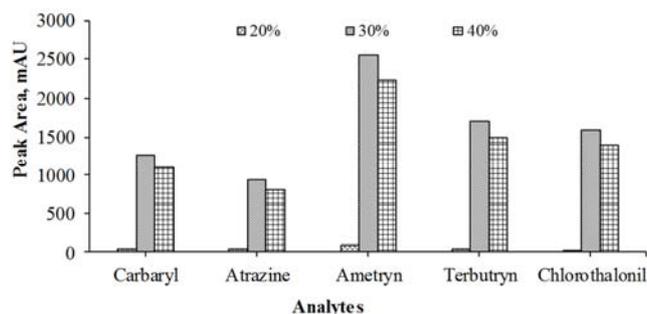(Figure 3). So, 30%; m/v MgSO4 was chosen as the optimum concentration for the subsequent studies.

Figure 3. *Effect of salt concentration on SALLE efficiency. Experimental conditions are mentioned in Figure 2.*

3.1.3. Effect of Acetonitrile Volume

The volume of acetontrile, the extraction solvent, as well plays a great role on the extraction performance of the SALLE procedure [31, 37, 42]. The effect of the acetonitrile volume on the extraction performance was investigated by varying its volume over the range of 0.5–2.0 mL. It was observed that the peak areas of the target analytes increased with the volume of the acetonirile from 0.5 to 1.0 mL and then decreased at higher volumes. At low volumes, phase separation between acetonitrile and aqueous phases was not clear and thus, collection of the organic phase was difficult. On the other hand, at higher volumes, the volume of organic phase obtained after phase separation is higher than its initial volume, indicating the existence of dissolved water in the organic phases [37, 42]. Based on the experimental results, 1 mL acetonitrile was selected as the optimum volume in the subsequent experiments.

3.1.4. Effect of the Sample pH

For efficient extraction of ionizable and relatively polar compounds, pH of the sample solution plays a decisive role. The sample solution pH should be lower than the pK$_a$ of the analytes to obtain the target analytes in their unionized forms so that they have higher tendency to partition into the organic phase [9, 43]. A series of experiments were performed to investigate the effect of pH on the extraction efficiency of the SALLE method for the target analytes by adjusting the pH of the sample solution over the range of 4–8, keeping other experimental parameters constant. As shown in Figure 5, peak areas of all the target analytes increased with the rise in pH of the sample solution up to pH 7 and then started to decrease on further increase in pH of the sample solution. The lower peak areas observed at higher pH might be most likely due to the hydrolysis of the pesticides [37]. On the other hand, the lower peak areas of the target analytes at lower pH might be attributed to their incomplete conversion to their neutral form and thus, complete transfer of the analytes from the sample solution to the organic phase could not be achieved. Therefore, a sample solution of pH 7 was chosen as the optimum.
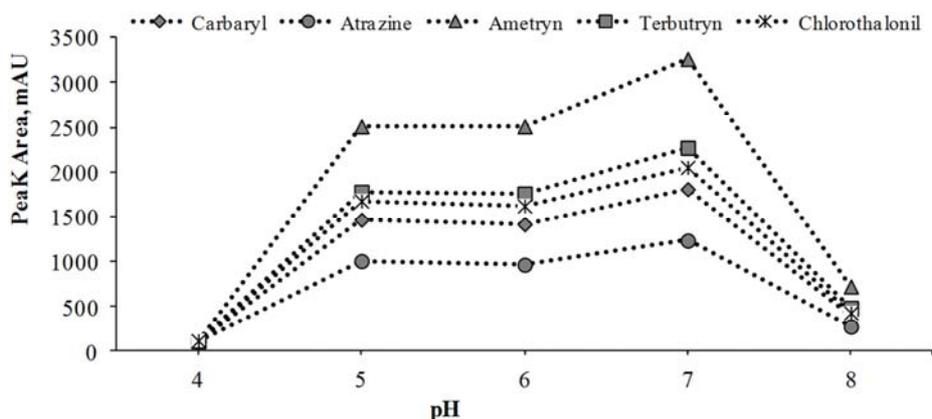
Figure 4. *Effect of sample pH on SALLE efficiency. Experimental conditions: salt concentration, 30% (m/v); and others are mentioned in Figure 2.*

3.2. Validation of the Proposed Method

3.2.1. Calibration Curves and Analytical Performance Characteristics

The proposed method was evaluated by constructing matrix-matched calibration curves using the beer sample as a representative matrix. The calibration curves were established by analyzing the extract of the spiked beer sample with the mixture of the analytes at six different concentration levels. Each level was extracted in duplicate and each extract was then injected in duplicate. Then, calibration curves were obtained by considering the average peak areas as the instrumental response versus the analytes concentrations. The coefficients of determination (R^2) for all the analytes were higher than 0.997, indicating good linearity over the studied concentrations range. The limits of detection (LOD) and quantification (LOQ) were determined as the minimum analytes concentrations yielding 3 and 10 times the signal-to-noise (S/N) ratio, respectively. The performance characteristics of the proposed SALLE method are shown in Table 1.

3.2.2. Precision Study

The precision of the method was studied in terms of repeatability (intra-day) and intermediate (inter-day)

precisions by extracting the spiked beer sample, at two concentration levels: Level 1: 50 µg L^{-1} and Level 2: 200 µg L^{-1}. Repeatability of the proposed method was investigated by extracting two spiked beer samples at both concentration levels and the obtained extracts were then injected in triplicate on the same day, under the same experimental conditions. Similarly, the intermediate precision of the method was also assayed by extracting one spiked beer sample at both concentration levels for three consecutive days. Each extract was then injected in triplicate. The results of both repeatability and intermediate precisions, which were expressed as the relative standard deviations (%RSD) of the peak areas are shown in Table 2. Acceptable precisions, i.e., %RSD less than 10.0, were obtained in all cases.

Table 1. *Statistical and performance characteristics of the proposed SALLE method.*

Analytes	Linear range (µg L^{-1})	R^2	LOD (µg L^{-1}.)	LOQ (µg L^{-1})
Carbaryl	9-250	0.998	2.6	8.6
Atrazine	11-250	0.997	3.3	10.9
Ametryn	5-250	0.997	1.3	4.5
Terbutryn	9-250	0.997	2.8	9.3
Chlorothal	13-250	0.999	3.9	12.8

Table 2. *Repeatability and intermediate precision of the proposed method (%RSD) for the spiked beer samples.*

Analytes	Repeatability (n = 6)		Intermediate precision (n = 9)	
	Level 1	Level 2	Level 1	Level 2
Carbaryl	6.6	0.8	7.6	9.2
Atrazine	6.2	1.3	9.3	8.7
Ametryn	4.0	4.6	9.1	9.3
Terbutryn	3.6	1.0	3.7	9.9
Chlorothalonil	7.8	1.6	4.8	9.8

3.2.3. Applications and Recovery Studies

The applicability of the developed method was investigated by performing recovery studies using three different types of alcoholic beverages including beer, wine and *Tej* samples. For recovery studies, each sample was spiked at two concentration levels previously used for precision studies. Each concentration level was extracted in duplicate and each extract was injected in triplicate. In all cases, blank samples were extracted and analyzed by the

proposed method; however, none of the target analytes was detected in these samples. Recoveries were calculated by comparing the concentration of the extracted analytes with the initial concentration of the target analytes spiked to the alcoholic beverage samples [44]. Recoveries and the corresponding %RSD (n = 6) of each target analyte in beer, wine and *Tej* samples are shown in Table 3. The observed recoveries were in the range of 71–104% in all the samples. These results were in good agreement with the acceptable

recovery range (i.e., from 70 to 120%) established by the European Commission for pesticide residue analysis in food

and feed samples [45].

Table 3. *Recovery values (%) of the method for the spiked alcoholic beverages.*

Recovery (%RSD; *n* = 6)		Carbaryl	Atrazine	Ametryn	Terbutryn	Chlorothalonil
Beer	Level 1	104 (1.3)	71 (1.6)	80 (1.5)	89 (6.5)	87 (2.1)
	Level 2	89 (2.3)	74 (0.9)	77 (1.2)	73 (3.6)	71 (5.2)
Wine	Level 1	101 (4.9)	90 (5.7)	78 (4.3)	88 (5.8)	79 (7.8)
	Level 2	86 (4.0)	72 (2.6)	74 (4.1)	71 (3.8)	72 (4.1)
Tej	Level 1	95 (5.0)	82 (9.2)	99 (3.1)	75 (7.3)	87 (6.0)
	Level 2	91 (5.9)	83 (8.9)	93 (2.5)	87 (3.0)	76 (4.0)

3.2.4. Selectivity of the Analytical Technique

Selectivity of the proposed method was evaluated by comparing the chromatograms of the unspiked alcoholic beverage samples with that of the corresponding spiked samples. Figure 5 shows representative chromatograms of the unspiked and spiked *Tej* samples with 50 μg L^{-1} of the target

pesticides. As can be seen from the chromatograms, no interferences were observed at the retention time of the target analytes, indicating that the proposed method has good selectivity for trace level analysis of the selected pesticides in alcoholic beverages and other related matrices.
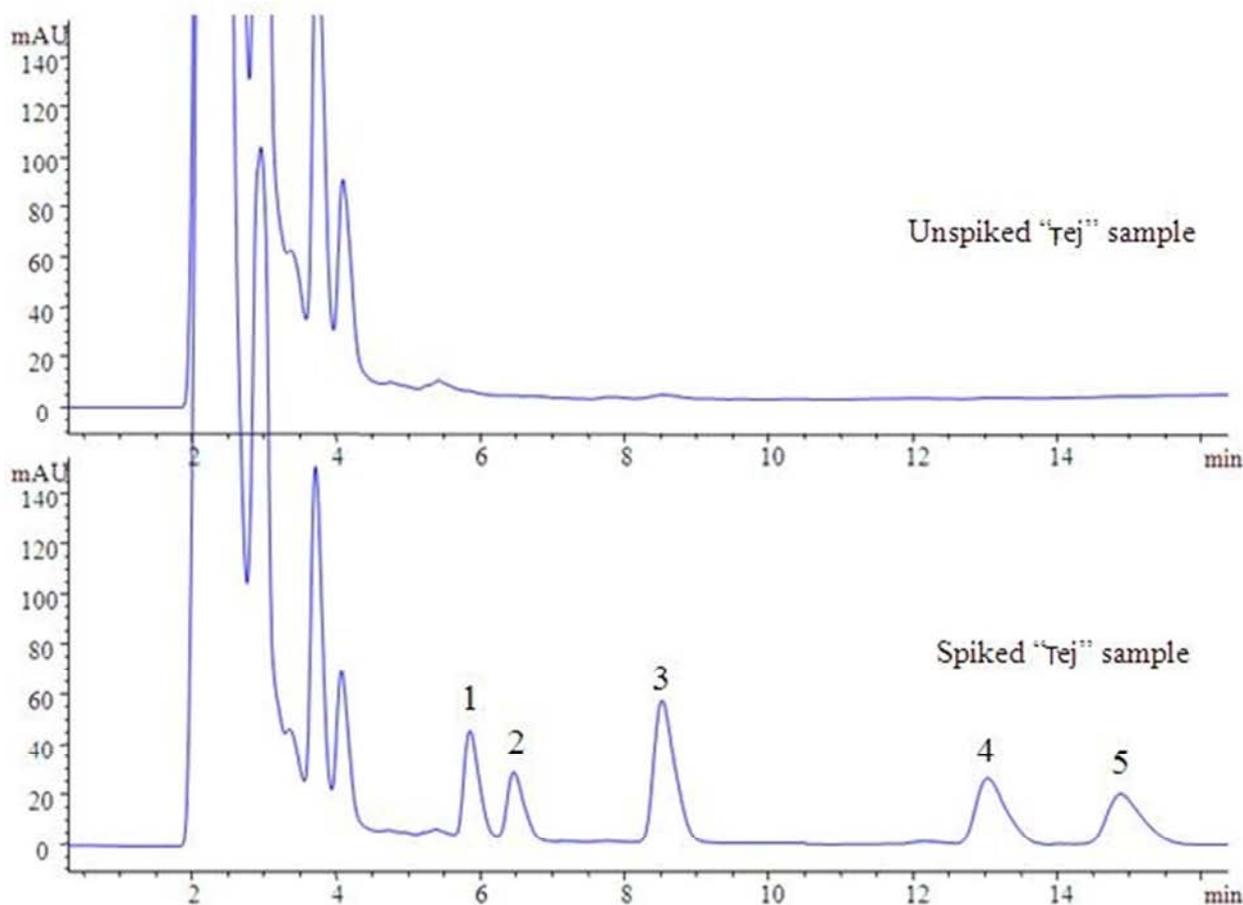

Figure 5. *The chromatograms of unspiked and spiked Tej samples showing the selectivity of the proposed method (1 = Carbaryl, 2 = Atrazine, 3 = Ametryn, 4 = Terbutryn, 5 = Chlorothalonil).*

4. Conclusions

In this study, a method that makes use of SALLE technique combined with HPLC-DAD has been proposed for determination of five multiclass pesticide residues in three different alcoholic beverages. Different parameters affecting the extraction performances of the target analytes such as the

salt type and its concentration, acetonitrile (extraction solvent) volume and pH were investigated and the optimum conditions were established. Using the optimum conditions, the proposed method exhibited its usefulness for the analysis of the target analytes, with good LODs and LOQs, good selectivity, acceptable precisions, wide linearity ranges and satisfactory recoveries. The method has advantages of simplicity, easy operation and short analysis time with

consumption of low volume of the less hazardous organic solvent, acetonitrile. Therefore, the developed method can be utilized as an attractive alternative for the determination of multiclass pesticides residues in various alcoholic beverages and other matrices.

Acknowledgements

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