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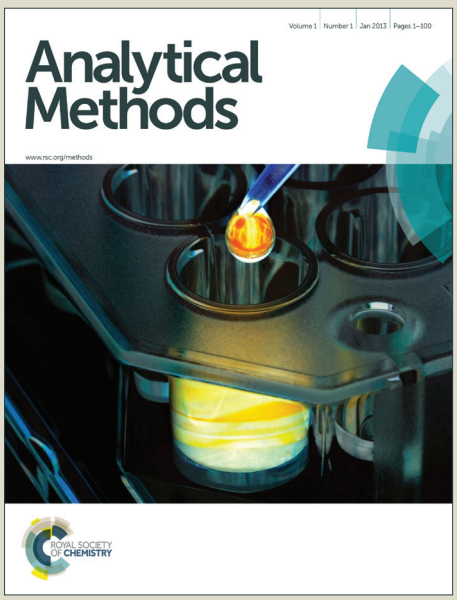

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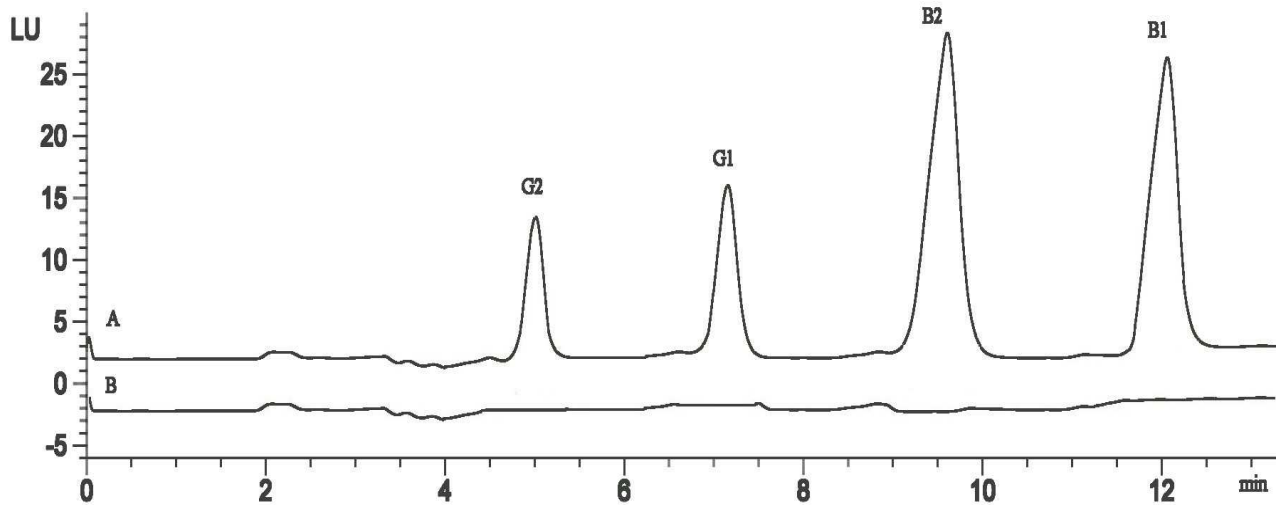

A novel method for high preconcentration of trace amounts of aflatoxins in pistachio by dispersive liquid-liquid microextraction after solid-phase extraction

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In the present study, a new approach which uses solid-phase extraction clean-up combined with dispersive liquid-liquid microextraction was proposed for the preconcentration of trace amounts of aflatoxins (B_1, B_2, G_1 and G_2). The aflatoxins were then determined using a high-performance liquid chromatography coupled with fluorescent detector. In this method, pistachio samples were extracted by ultrasound-assisted extraction followed by solid phase extraction. Then, the solid phase extract was used as disperser solvent of the next dispersive liquid-liquid microextraction step for further purification and enrichment of aflatoxins. The effects of various parameters on the extraction efficiency of the proposed method were investigated and optimized. Good linearity of aflatoxins was obtained from 0.1 to 50.0 μg kg^{-1} for B_1 and B_2 and from 0.2 to 50.0 μg kg^{-1} for G_1 and G_2, respectively. The limits of detection (LODs) (S/N=3) were 0.02 for B_1 and B_2 and 0.04 μg kg^{-1} for G_1 and G_2, respectively. The relative recoveries at the three spiked levels were ranged from 85 to 93% with RSD less than 13% (n=3). The method has been successfully applied to the determination of aflatoxins in pistachio samples.

1. Introduction

Aflatoxins (AFs) are bisfuranocoumarin compounds and members of a major group of mycotoxins produced as secondary metabolites by fungi, Aspergillus flavus and Aspergillus parasiticus. These mycotoxins are highly toxic and carcinogen and have been detected in various food commodities. Aflatoxins are normally refers to the group of difuranocoumarins and classified in two broad groups according to their chemical structure; the difurocoumarocyclopentenone series (AFB1, AFB2, AFB2A, AFM1, AFM2, AFM2A and aflatoxicol) and the difurocoumarolactone series (AFG1, AFG2, AFG2A, AFGM1, AFGM2, AFGM2A and AFB3). Although 18 different aflatoxins have been identified, the four most prevalent aflatoxins are aflatoxin B_1 (Af-B_1), aflatoxin B_2 (Af-B_2), aflatoxin G_1 (Af-G_1) and aflatoxin G_2 (Af-G_2) whose chemical structures are shown in figure 1. The Af-B_1 is listed as a carcinogen of group I by International Agency for Research on cancer.[1] Aflatoxins B_1 and B_2 produce a blue fluorescence where as G_1 and G_2 produce green fluorescence. Therefore, the contamination of food products such as cereals and Pistachio and the other commodities with these mycotoxins is controlled by legal limits (as maximum tolerated level, MTL).[2] The MTLs regulated by European Union (EU) are 2 and 4 μg kg^{-1} for Af-B_1 and total aflatoxin (Af-T), respectively, in groundnuts, nuts, dried fruits and cereal.[3] Pistachio is one of the food commodity classes with the highest risk of AF contamination.[2] Iran is as a major worldwide pistachio producer and Institute of Standards and Industrial Research of I.R. Iran (ISIRI) has set a MTL of 5 and 15 μg kg^{-1} for Af-B_1 and Af-T, respectively, in 2002.[4] Many research studies have been made on investigation of food and feedstuff contamination with mycotoxins[5-7] and in a recent study incidence of AF in Iran pistachio has been investigated.[8] Thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) with fluorescence detection are the most frequently used quantitative methods in research and routine analyses of aflatoxins.[9-14] Other

analytical techniques, which may be used in aflatoxin analysis, are enzyme-linked immunosorbent assay (ELISA), electrophoresis and gas chromatography-mass spectrometry.[15-17] Several clean-up methods for determination of mycotoxins, such as immunoaffinity columns, liquid-liquid extraction, supercritical fluid extraction and solid-phase extraction (SPE) methods were reviewed by Turner et al.[18] However, some of these conventional extraction methods are time-consuming, tedious, expensive and require large volume of toxic solvent, which is harmful to the environment and has some disadvantages such as possible loss of sample by adsorption onto glassware in liquid-liquid extraction method.[19,20] Association of Analytical Communities (AOAC) method of aflatoxin analysis is based on the extraction by immunoaffinity column and quantification by reversed-phase LC with post-column derivatization involving bromination.[21] Dispersive liquid-liquid microextraction (DLLME), a miniaturized extraction technique introduced in 2006,[22] was found to be extremely simple, quick, efficient, and with a very low consumption of solvents. This technique is based on a ternary component solvent system: aqueous sample or water, extraction and disperser solvents. The latter should be soluble in the extraction solvent and miscible in water, thus enabling the formation of cloudy solution and the quick extraction equilibrium. Since its introduction, DLLME has been applied for extraction of different compounds.[23-28]

Despite several advantages of DLLME, this method is not suitable for extraction of aflatoxins in pistachio. SPE-DLLME is an efficient hyphenated technique that offers the advantages both methods such as simplicity, low solvent usage and exposure, low disposal costs and extraction time, with high recovery and enrichment factor and it can be also used in complex matrices.[29-32]

In this work, SPE-DLLME followed by HPLC with fluorescence detection was used for the determination of aflatoxins in pistachio. Different parameters affecting the extraction process were studied and optimized in detail.

2. Experimental

2.1. Chemicals and reagents

Standard solutions of AFs containing 2 µg mL^{-1} (B_1, B_2, G_1 and G_2) were obtained from Sigma-Aldrich (Milwaukee, WI, USA). The daily standard working solutions of different concentrations were obtained by diluting the stock solutions with methanol/water. All solutions were kept at 4 ºC in the dark. Carbon tetrachloride, chloroform, chlorobenzene, carbon tetrachloroethylene, acetone, acetonitrile, methanol, ethanol and sodium chloride were obtained from Merck (Darmstadt, Germany). The water used was purified on a Nanopure ultra pure water purification system (Nano pure, USA). Since the city of Rafsanjan is the major pistachio producer in Iran, it was considered as the source of real samples and a number of five packs of Rafsanjan pistachio were purchased from a local market in Iran.

2.2. HPLC system

An Agilent 1100 series high performance liquid chromatography (HPLC) equipped with a vacuum degasser, a quaternary pump, an automatic sample injection system, an electrochemical cell for the post-column bromine derivatization of aflatoxins (Model KB LIbios cell, Libios, France), a fluorescence detector was used for the separation and determination of aflatoxins. Separation was carried out on a C-18 reverse phase column (C_{18}, 5 µm, 4.6mm × 25 cm column, Waters, USA) and the mobile phase water/methanol/acetonitrile (60:20:20 v/v), containing 119 mg of potassium bromide and 100 µL of 65% nitric acid, was pumped at a flow rate of 1 mL min^{-1}. The excitation and emission wavelengths were 360 and 440 nm, respectively.

2.3. Ultrasound-assisted extraction

In order to enhance the recovery and shorten extraction time, we used ultrasound-assisted extraction. Typically, appropriate amount of pistachio samples were minced using a kitchen homogenizer and blended to homogenize them. The optimization of the ultrasound-assisted extraction of aflatoxins from pistachio samples was developed with samples that free of aflatoxins. For this purpose, extraction of the spiked samples (10.0 μg kg^{-1} fortification level) was carried out with different mixtures of acetonitrile, methanol, acetone and water. Different amounts of samples (between 1.0 and 10.0 g) were sonicated with the solvents between 5 and 30 min. Results showed that the best recoveries were achieved using methanol as an extracting solvent. In general, the addition of the small amounts of water to methanol, acetone and acetonitrile as extracting solvents, improved the recoveries when compared to extractions carried out only with organic solvents. Among the different mixtures tested, the extraction of 5.0 g of the homogenized sample for 20 min with 20 mL of methanol: water 4:1 (v/v) was enough to provide a good extraction of aflatoxins. Different amounts of water were used for dilution the extracts. Extracts were collected and brought to 30, 60, 90 and 120 mL. The results show that when the 60 mL was used, the best recoveries were obtained and more dilution causes to decrease the extraction efficiency of the analytes especially for Af-G_1 and Af-G_2 (as the more polar compounds). Therefore, extract was collected and brought up to 60 mL with deionized water. This final test portion of 60 mL was passed through the C_{18} cartridges using the SPE procedure.

2.4. SPE-DLLME procedure

5.0 g of the homogenized sample was weighted in a 50 mL centrifuge tube and 20 mL of methanol: water 4:1 (v/v) and 10 mL of n-hexane and 1.0 g of NaCl was added. Pistachio extracts were quite dirty because of the hydrophobic co-extracted matrix components. These types of matrix interferences are very severe for HPLC analysis; therefore, the elimination of lipids from pistachio extract is necessary. N-hexane was used for de-fatting pistachio extract. Ultrasound

assisted extraction was carried out for 20 min using a 40 kHz and 0.138 kW ultrasonic water bath with temperature control (Tecno-Gaz SpA, Italy). Samples were centrifuged for 4 min at 5000 rpm. The extracts were filtered on a filter paper (Whatman No 44) and then supernatant solution was centrifuged for 6 min at 5000 rpm. After separation of the two phases by centrifugation, n-hexane was eliminated and the lower phase was used for the SPE process. The extract was collected and brought up to 60 mL with deionized water. The final extract (60 mL) was transferred to a C_{18} sorbent (3 mL syringe barrel, waters, USA), which activated with 5 mL of methanol followed by 5 mL of methanol: water (4:1 v/v), previously. After loading the sample into the SPE at a low rate of about 6.7 mL min^{-1} with the aid of a vacuum pump (Rotavac, Heidolph, Germany), it was dried. Aflatoxins were eluted with 1.5 mL methanol and was collected into the test tube and was used as disperser solvent in the subsequent DLLME procedures. 5.0 mL aqueous solution was placed in a 10 mL screw cap glass test tube with conical bottom. 1.5 mL methanol (disperser solvent) containing 200.0 μL chloroform (extraction solvent) was injected into the aqueous solution, using a 5.0 mL syringe (gas tight, Hamilton, Reno, NV, USA). A cloudy solution, resulting from the dispersion of the fine chloroform droplets in the aqueous solution was formed in the test tube. In this step, aflatoxins extracted into the fine chloroform droplets in a few seconds. The mixture was then centrifuged for 3 min at 5000 rpm. After this procedure, the dispersed fine chloroform droplets were sedimented at the bottom of the conical test tube (about 25 μL). The sedimented phase was completely transferred to another test tube with conical bottom using 50 μL HPLC syringe and after evaporation of the solvent in a water bath; the residue was dissolved in 30 μL HPLC grade methanol and injected into the separation system.

3. Results and discussion

In this research, SPE-DLLME method combined with HPLC-FL was developed for the determination of aflatoxins in pistachio samples. The combination of SPE and DLLME not only resulted in a high enrichment factor, but also it could be used in complex matrices (pistachio samples) to reduce matrix effects on the extraction and determination steps. In order to obtain the best extraction performance, different parameters affecting the extraction process were studied and optimized.

3.1. Effect of type and volume of the extraction solvent

Careful attention should be paid to the selection of the extraction solvent. The extraction solvent must have some properties, such as higher density than water, high extraction capability of the analytes and low solubility in water. Carbon tetrachloride (CCl_4), carbon tetrachloroethylene (C_2Cl_4), chlorobenzene (C_6H_5Cl) and chloroform ($CHCl_3$) was examined in this study. A series of the sample solutions were tested using 1.5 mL methanol, containing different volumes of the extraction solvents to achieve about 25 µL volume of the sedimented phase. Thereby, 200.0, 55.0, 52.0 and 57.0 µL of $CHCl_3$, C_6H_5Cl, C_2Cl_4 and CCl_4 were used, respectively. The results (Fig. 2) indicate that the $CHCl_3$ has the highest extraction efficiency in comparison with the other tested solvents. It is probably, because of the higher solubility of aflatoxins in the $CHCl_3$ in comparison with the other tested solvents. Therefore, $CHCl_3$ was selected as the main extraction solvent.

In order to examine the effect of the extraction solvent volume, 1.5 mL of methanol containing different volumes of $CHCl_3$ (170.0, 200.0, 230.0, 260.0, 290.0 and 320.0 µL) was subjected to the same SPE-DLLME procedures. By increasing the volume of $CHCl_3$ from 170.0 to 200.0 µL, the peak area of aflatoxins increases, but by increasing the volume of $CHCl_3$ from 200.0 to 320.0 µL, the peak area of aflatoxins decreases (Fig. 3). Because the concentration of the analytes in the

sedimented phase decreases and dilution effect. Therefore, 200.0 µL of $CHCl_3$ was selected as the optimum volume of the extraction solvent.

3.2. Effect of type and volume of disperser solvent

When combining SPE with DLLME, the elution solvent of SPE should also play the role of the disperser solvent at the DLLME stage. The main criterion for selecting the disperser solvent is its miscibility with the extraction solvent and the aqueous sample. For this purpose, different solvents such as acetonitrile, acetone, ethanol and methanol were examined. A series of sample solutions were tested using 1.5 mL of each disperser solvent, containing 200.0 µL volume of $CHCl_3$ (as extraction solvent). The results (Fig. 4) indicated that methanol has the highest peak area in comparison with the other tested solvents. Thus, methanol was chosen as the eluent and disperser solvent for subsequent experiments.

In order to examine the effect of the disperser solvent volume, the volume of the sedimented phase was kept constant (25 µL) and the volume of methanol and $CHCl_3$ was changed, simultaneously. The different volumes of methanol (0.5, 1.0, 1.5 and 2.0 mL) were in concomitant with the corresponding volumes of 175.0, 188.0, 200.0 and 215.0 µL of $CHCl_3$, respectively. It was obvious from figure 5 that, 1.5 mL of methanol has the highest peak area than that of the others. Therefore, 1.5 mL of methanol was selected as the optimum volume of disperser solvent.

3.3. Effect of the flow rate of the sample solution

The flow rate of the sample solution through the solid phase is an important factor, because it controls the time of analysis. The flow rate, on the one hand, must be low enough to perform an effective retention of the analytes. On the other hand, it must be high enough not to waste time. The flow rate influence of the sample solutions from the solid-phase cartridge on the aflatoxins

recovery was investigated in the range of 0.65-8.6 mL min^{-1}. It was found that in the range of 0.65-6.7 mL min^{-1}, the aflatoxins recovery by the cartridge was not affected considerably by the sample solution flow rate (Fig. 6). According to the result, 6.7 mL min^{-1} was used as the best sample flow rate.

3.4. Effect of salt addition

The influence of the ionic strength was evaluated at the concentration levels of 0-8% (w/v) of NaCl while other parameters were kept constant. The experimental results show that salt addition had no significant effect on the extraction efficiency of the analytes. Therefore, all the following experiments were carried out without addition of salt.

3.5. Analytical performance

The figures of merit of the proposed method are shown in table 1. The calibration curves were made under the optimized conditions using the samples free of the aflatoxins spiked at the different concentrations of the target analytes. The calibration curves showed a satisfactory linearity within the concentration range: 0.1-50.0 µg kg^{-1} for B_1 and B_2 and 0.2-50.0 µg kg^{-1} for G_1 and G_2; and the coefficient of estimation (r^2) 0.9984 for B_1 and 0.9991 for B_2 and 0.9989 for G_1 and 0.9975 for G_2. Based on signal-to-noise ratio (S/N) of 3, the limits of detection (LODs) was 0.02 µg kg^{-1} for B_1 and B_2 and 0.04 µg kg^{-1} for G_1 and G_2, respectively, which was below the maximum residue limits. Precision expresses as the relative standard deviations (RSDs, n=5) were 6.5, 7.2, 7.4 and 8.6%, for B_2, B_1, G_1 and G_2, respectively. For consideration the effect of the DLLME method on the quantitative results, the proposed method was done without the DLLME method which means that methanol got from the SPE method was injected into the HPLC directly. The results show that the calibration curves within the concentration range: 5.0-50 µg kg^{-1} for B_1 and B_2 and 10.0-50.0 µg kg^{-1} for G_1 and G_2.

3.6. Analysis of samples

The chromatograms of the pistachio samples before spiking and after spiking at a 2.5 μg kg^{-1} concentration level of the aflatoxins are shown in Fig. 7. To study the effect of the sample matrix and the accuracy of the SPE-DLLME-HPLC method, recovery experiments were carried out by spiking the three levels of the aflatoxins in the samples (Table 2). The relative recoveries for the aflatoxins at the three spiked levels were in a range of 85% - 93 % with RSD less than 13% (n=3), which indicated that the method was reliable and could be used for the determination of trace amount of the aflatoxins in the pistachio samples. The comparison of the proposed method with other reported methods immunoaffinity column[33] and solid-phase extraction[34] demonstrated that SPE-DLLME-HPLC-FL method has a wide linear range, lower detection limit, higher preconcentration factor and short extraction time. The proposed method is easy to operate in the extraction and the determination of aflatoxins.

4. Conclusions

In this study, a rapid and simple analytical procedure has been successfully developed for the analysis of aflatoxins in pistachio samples. The method provides useful information about the risk of AF hazard in pistachio products in Iran. To the best of our knowledge, this is the first time that SPE-DLLME has been applied for the preconcentration and determination of aflatoxins in pistachio samples, and it displayed wide linearity, good precision, and satisfactory relative recoveries. The proposed method also eliminates the use of immunoaffinity columns encountered in ELISA and gives a LOD that is either better or competitive with current methods. We are convinced that the technique possesses a great potential in rapid preconcentration and analysis of the aflatoxins from pistachio samples.

Acknowledgements

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Figure Captions

Fig.1. Chemical structures of the aflatoxins B_1, B_2, G_1 and G_2

Fig.2. Effect of the type of the extraction solvent on the extraction efficiency. Extraction conditions: disperser solvent (methanol) volume,1.5 mL; extraction solvent volumes, 200.0 µL $CHCl_3$, 55.0 C_6H_5Cl, 57.0 CCl_4, 52.0 C_2Cl_4; flow rate, 6.7 mL min^{-1}.

Fig.3. Effect of the extraction solvent ($CHCl_3$) volume on the peak area of the analytes which obtained from SPE-DLLME. Extraction conditions: disperser solvent (methanol) volume, 1.5 mL; extraction solvent ($CHCl_3$) volumes, 170.0, 200.0, 230.0, 260.0, 290.0 and 320.0 µL; flow rate, 6.7 mL min^{-1}.

Fig.4. Effect of the type of the disperser or eluent solvent on the extraction efficiency. Extraction conditions: disperser solvent (acetone, acetonitrile, ethanol and methanol) volume, 1.5 mL; extraction solvent ($CHCl_3$) volume, 200.0 µL; flow rate, 6.7 mL min^{-1}.

Fig.5. Effect of the disperser solvent (methanol) volume on the peak area of the analytes which obtained from SPE-DLLME. Extraction conditions: disperser solvent (methanol) volumes, 0.5, 1.0, 1.5 and 2.0 mL; extraction solvent ($CHCl_3$) volumes, 175.0, 188.0, 200.0 and 215.0 µL; flow rate, 6.7 mL min^{-1}.

Fig.6. Effect of the flow rate on the peak area of the analytes which obtained from SPE-DLLME. Extraction conditions: disperser solvent (methanol) volume, 1.5 mL; extraction solvent ($CHCl_3$) volume 200.0 µL.

Fig.7. HPLC chromatograms of (B) before spiking with the analytes in the pistachio samples, (A) 2.5 µg kg^{-1} spiked of the analytes in the pistachio sample after extraction via the proposed method at the optimum conditions.

Table 1

Figures of merit of the procedure

Analytes	LOD[a] (µg Kg^{-1})	RSD[b] (%)	Linear range (µg Kg^{-1})	r^2
B_1	0.02	7.2	0.1-50.0	0.9984
B_2	0.02	6.5	0.1-50.0	0.9991
G_1	0.04	7.4	0.2-50.0	0.9989
G_2	0.04	8.6	0.2-50.0	0.9975

[a] Limit of detection on the based of S/N=3

[b] Relative standard deviation, n =5

Table 2

Determination of aflatoxins (B_1, B_2, G_1 and G_2) in pistachio samples

Spiking level ($\mu g\ kg^{-1}$)	Concentration of B_1, B_2, G_1 and G_2 ($\mu g\ kg^{-1}$)				RSD (%) , n=3				Relative recovery (%)			
	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2	B_1	B_2	G_1	G_2
1.0	n.d[a].	n.d[a].	n.d[a].	n.d[a].	11.2	8.8	10.5	12.5	89	91	87	85
2.5	n.d.	n.d.	n.d.	n.d.	10.6	7.4	8.7	11.1	91	93	89	87
5.0	n.d.	n.d.	n.d.	n.d.	9.8	7.8	10.3	10.7	90	92	88	86

[a] Not detected.

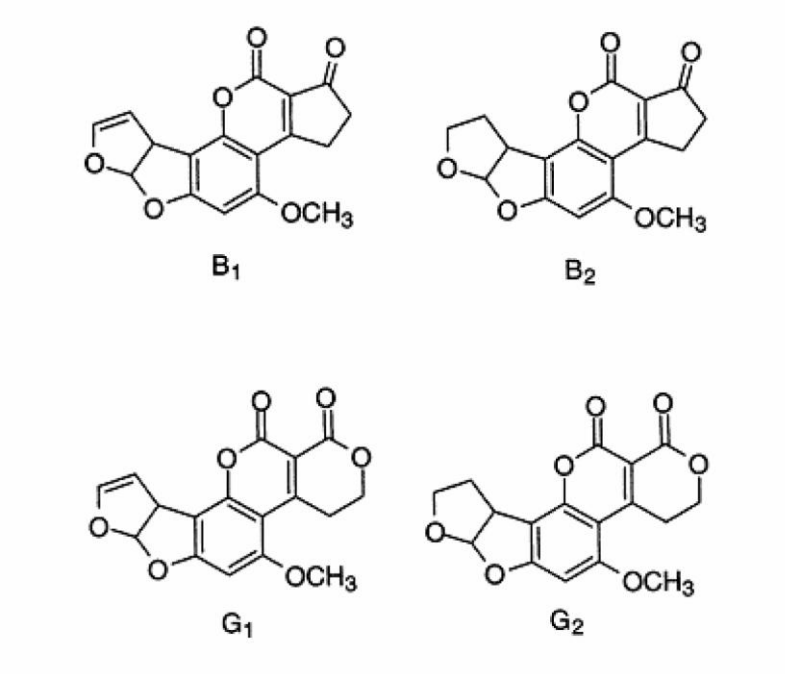

Figure 1

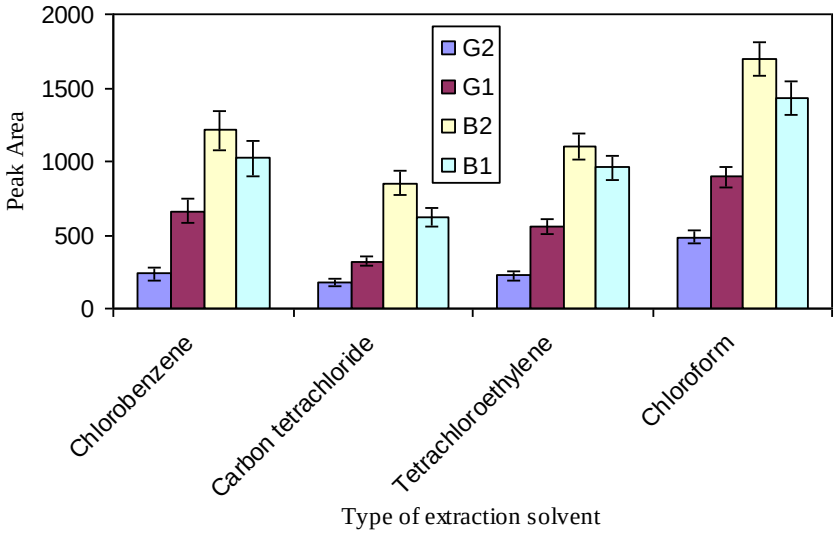

Figure 2

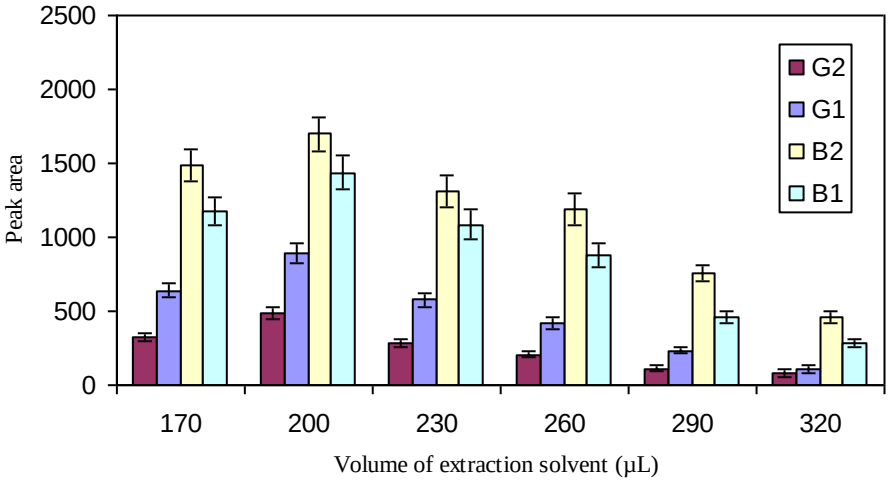

Figure 3

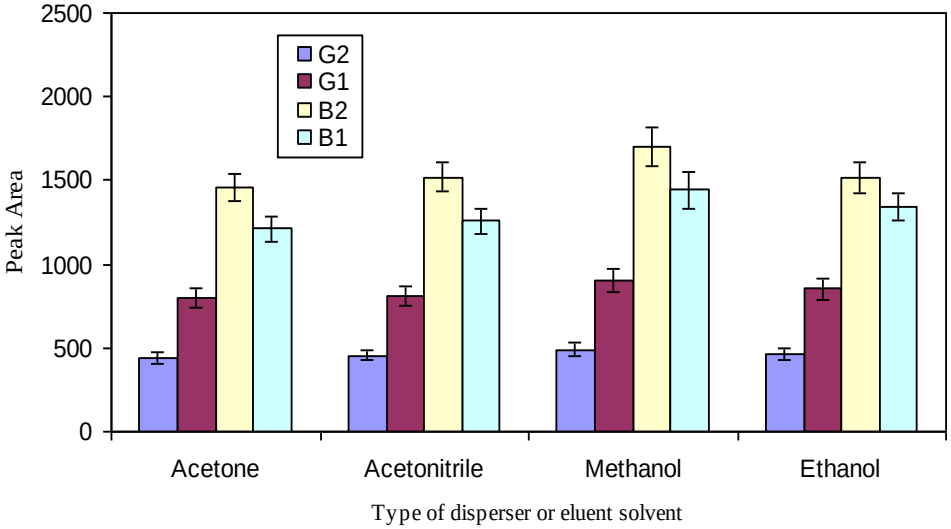

Figure 4

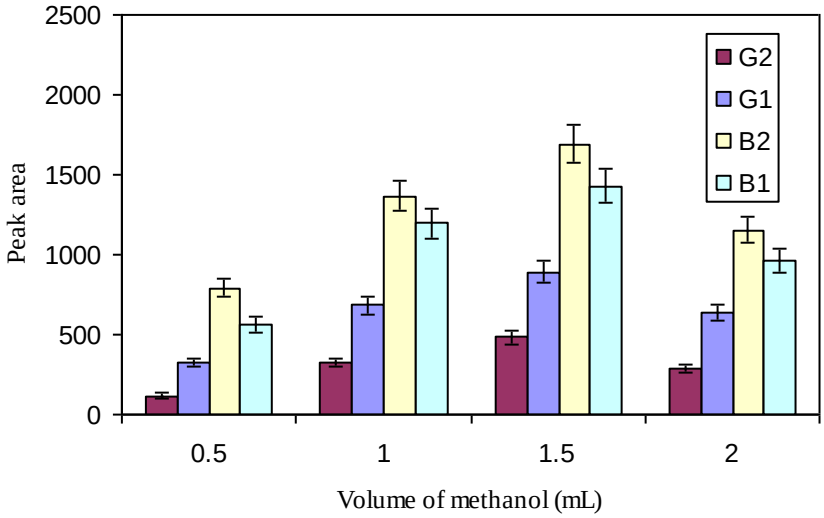

Figure 5

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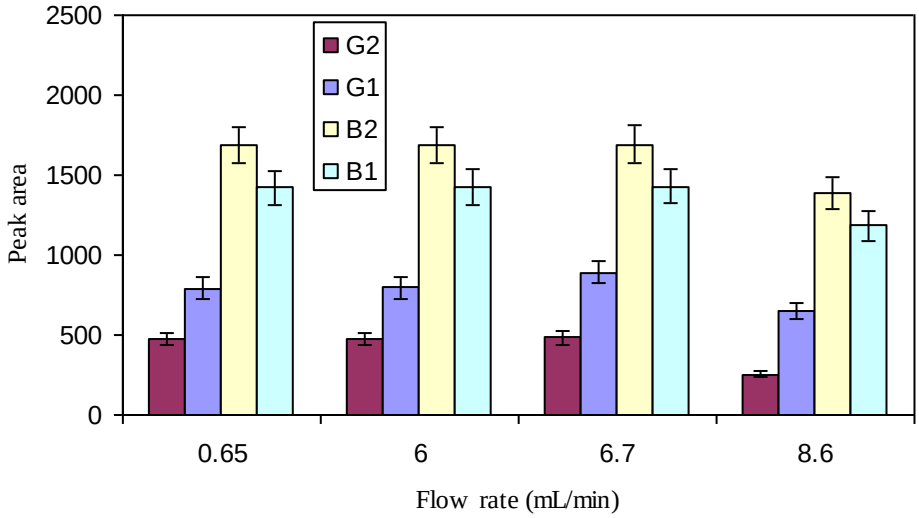

Figure 6

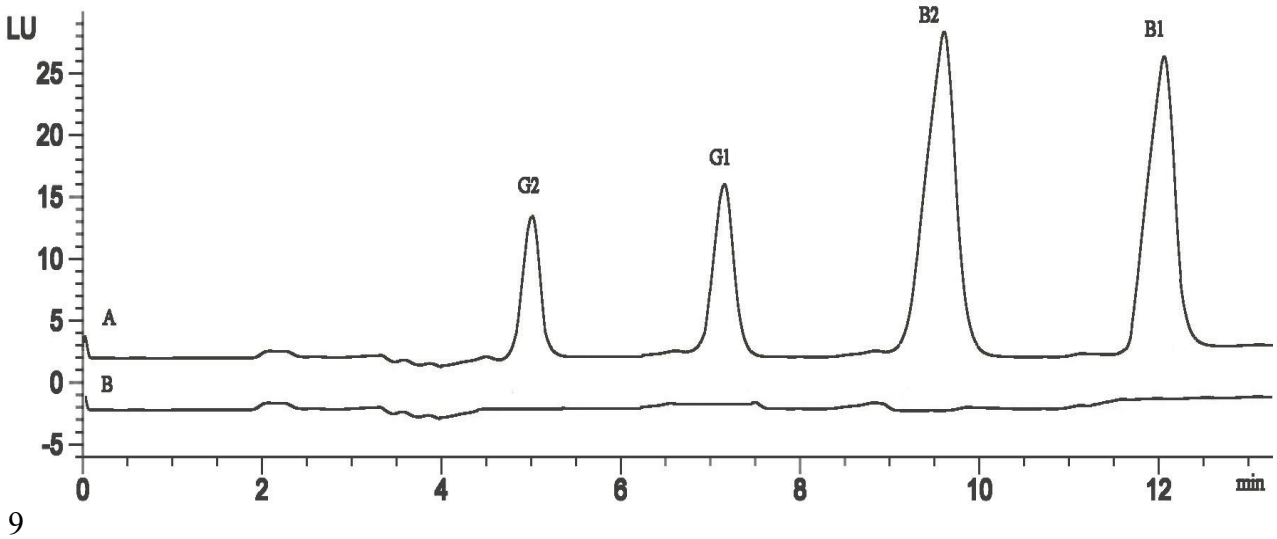

Figure 7