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Research Article

Extraction of Cyproheptadine as Potent Appetizing Stimulant in Herbal Supplements by Efficient Carbon Nitride Nanosheets as Dispersive Solid Phase Extraction Adsorbent

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Abstract

Background: Application of natural-based herbal medicine is on a growing trend in some countries and people prefer to use plant-originated drugs rather than chemical-based ones. The present study describes an interesting sample preparation method for extraction and determination of cyproheptadine in herbal supplements as appetizing stimulant by using carbon nitride nanosheets as dispersive solid phase extraction method coupled with HPLC-UV.

Methods: Various techniques used for characterization of adsorbent such as: Infrared spectroscopy (IR), scanning electron microscopy (SEM), Zeta potential analysis and powder X-ray diffraction (XRD). Optimization of the important extraction parameters were conducted by one parameter-at-a time method. Next, method validation was carried out.

Results: The optimized cyproheptadine extraction parameters were introduced and under optimized conditions the method presented a good linearity in the concentration range of 300-2000 ng/g. The limit of detection (LOD) was 100 ng/g for the introduced method.

Conclusion: Quantitative analysis of fifteen real samples (Tablets or capsules) by proposed method confirmed the illegal presence of cyproheptadine in herbal appetizing stimulants supplements of the markets.

Introduction

Application of natural-based herbal medicine is on a growing trend in some countries and people prefer to use plant-originated drugs rather than chemical-based ones. People are using plant-originated drugs for various purposes for instance, enhancing sexual performance, bodybuilding, obesity and many other problems.[1-4] Therefore, different types of capsules, tablets or syrups are available in the market claiming to be herbal-based which are suspected to contain adulterant or undeclared synthetic drugs. Cyproheptadine hydrochloride is classified as antihistamines, extensively used as a stimulating agent for appetizing in underweight children and also adults. Also, it is an antiserotonergic agent, presenting inhibitory activities for l-type calcium channels.[5,6] Herbal supplements labelled as appetite stimulant and sold in the market must be proved to possess certain degree of safety and efficiency. For this purpose, these herbal products must be approved under the supervision of food and drug administration.[7,8] Unfortunately, some local herbal markets do not obey this rule and distribute hand-made herbal products which are not safe and efficient. Moreover, these products might include chemical and synthetic ingredients that are sold as plant-originated ones used for appetizing especially for children in the growth age. This strategy develops their business.[9] Accordingly, analysis of adulterated herbal supplements is an interesting field in forensic toxicology, quality control and analytical chemistry. Sample preparation is an important step in analytical chemistry due to its low analyte concentration and matrix effect which are influential parameters regarding the sensitivity and selectivity of an analysis method.[10,11] Solid phase extraction (SPE) is an influential sample preparation method which is employed for extraction of a wide variety of the analytes from aqueous or biological medias.[12,13] SPE is introduced as an alternative method to liquid-liquid extraction (LLE) extraction method.[1,14] Dispersive solid phase extraction (DSPE) is an efficient SPE-based method which is green, simple and minimized with a lower amount of adsorbent (lower than 500 mg).[15-17] Besides, DSPE is fast and cost-effective, and avoids high back pressure.[18,19] This method

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was first introduced by Anastadiades et al. for extraction of pesticides.[20] DSPE usually consists of four steps as follows: a) direct dispersion of the adsorbent in the media b) extraction of the analyte c) separation of the adsorbent from the media d) proper solvent elution.[21] Use of various nanoparticles in DSPE adsorbent structure significantly improves the interactions between the analyte and the adsorbent due to its high surface area-to-volume ratio which provides an efficient analyte transfer to adsorbent, and then a higher extraction efficiency is obtained.[13,22] Additionally, modification of nanoparticles makes hydrophilic functional groups on the nanoparticles surface, which leads to a sufficient dispersion of the adsorbent in the sample matrix.[15] Graphitic carbon nitride nanosheets (CNNs) are the novel class of carbon based materials with unique properties. CNNs present an extended delocalize π electron system which persuades a good π-interaction towards compounds with aromatic benzene ring in their structure.[23] A strong covalence C-N band exists in each layer consisting of tri-s-tirazine which are connected via tertiary amines. The Vander Waals interaction exists between layers. Two sides of the poly aromatic scaffold of the planar sheets are accessible for adsorption of the analyte from the media.[24,25] CNNs are commonly synthesized by facial and cost-effective pyrolysis of precursors containing carbon and nitrogen like melamine, thiourea, urea, cyanamide and dicyandiamide without applying organic solvents.[26] The interesting structural properties of the CNNs encourage its application in different fields like hydrogen storage, electro-generated chemiluminescence, photocatalysis, lithium-ion battery and solid phase micro extraction (SPME).[27-30] In this study CNNs were synthesized by a simple pyrolysis of urea and after full characterization, they were employed in DSPE process of cyproheptadine extraction from herbal supplements provided from the markets claiming to provide natural herbal supplements and cyproheptadin free products as appetizing stimulant.

Materials and Methods

Reagents

Cyproheptadine hydrochloride was kindly donated from Amin pharmaceuticals Co., (Isfahan, Iran). Ammonium acetate, urea, HCl, NaOH, were provided from Merck Chemicals (Darmstadt, Germany). Acetone, methanol, acetonitrile (HPLC grade) were purchased from Merck Chemicals (Darmstadt, Germany). Fifteen herbal supplements were purchased from local herbal shops (Tabriz, Iran). Ultrapure water was obtained from Milli-Q water system (Darmstadt, Germany).

Instruments

Zeta potential measurements were performed by Zetasizer (Nanotrac Wave, Microtrac, Germany). Powder X-ray diffraction patterns (XRD) were conducted by D5000 (Siemens, Germany) instrument. Infrared spectroscopy was recorded by Tensor 27 FTIR instrument (Bruker, Germany). Scanning electron microscopy (SEM) (MIRA3 FEG–SEM, Tescan, the Czech Republic) was applied. High performance liquid chromatography (HPLC) analysis was carried out by Knauer (Germany) system equipped with a UV–visible detector (K-2600, Knauer, Germany). Analytical C18 column (5 µm particle diameter, 4.6 mm i.¬ d. × 25 cm) (Knauer, Germany) was applied for separation at room temperature.

Sample preparation

Herbal supplements were provided from different herbal shops randomly. They were as tablets or capsules dosage forms. The shell of the capsules was removed and the powder was homogenised. Then, the samples were smashed and 0.1g of each sample was transferred into 5 mL vial and applied for the DSPE extraction process.

Synthesis of CNNs extraction adsorbent

The synthesis of CNNs was carried out by a single-step process. For this purpose, 20 g of urea was pyrolyzed in 550 °C oven Behdad (Iran) for 4 h by heating rate of 4°C min -1. Afterwards, the obtained yellowish powder was washed with deionized water three times and then dried at 60 °C. The product was applied for DSPE process.[26]

DSPE extraction process

Aqueous blank solution was prepared as follows: 0.1 g of smashed herbal sample (which was bought from local herbal shops and was studied to know whether it is blank) was weighed and dispersed thoroughly in 5 mL double distilled water. Afterwards, the sample was centrifuged for 10 minutes and the supernatant was separated. Later, 20 µL of the supernatant was injected into HPLC system to distinguish the blank sample which is cyproheptadine free one. After finding one cyproheptadine free sample, it was applied as the blank matrix for DSPE extraction process. The extraction process was implemented as follows: 20 mg of CCNs was weighed and dispersed in 5 mL of aqueous solution and spiked with 0.5 µg/mL cyproheptadine (pH=8). The sample was stirred for 5 minutes with the 4000 stirring rate (rpm) and then was centrifuged and the supernatant was thrown away. Next, 200 µL of acetonitrile was used as desorption solvent while the sample was sonicated for 3 minutes. After centrifuging, 20 µL of desorption solvent was loaded into the HPLC system. Acetonitrile-ammonium acetate buffer consisting of 45:55 (V/V), (5 mM, pH=5.5) was selected as mobile phase at the flow rate of 1 mL/min with slight modification.[31] The 827 pH lab meter was used for pH adjustment from Metrohm (Switzerland).

Results and Discussion

Characterization of synthesized CNN

Figure 1a presents the IR spectroscopy of the CNNs. A peak at 809 cm -1 and 1245-1640 cm -1 are related to the breathing mode of tirazine and the stretching mode of C-N heterocycles, respectively. A broad peak at 3000-3500 cm^{-1} is associated with the vibration of N-H band. The

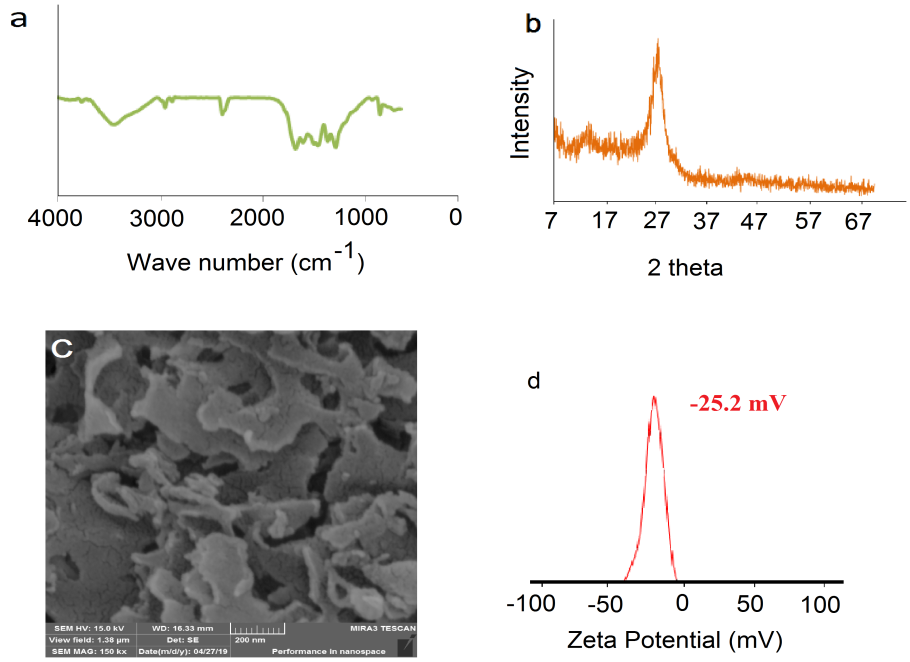

Figure 1. a) Infrared spectroscopy (IR) of the CNNs, **b)** X-ray diffraction (XRD) pattern of the synthesized CNNs, **c)** Scanning electron microscopy (SEM) image of CNNs and **d)** Zeta potential value.

obtained results are in a good accordance with previous published papers.[32,33] The X-ray diffraction pattern (XRD) of the synthesized CNNs is represented at (Figure 1b). The presence of peak at 2Θ = 12.6° is correspondence to the in-planar structural packing motif of the CNN common structure. A sharp peak at 2Θ = 27.08° is related to the stacking of the conjugated aromatic system as in the graphite. The results are in agreement with the literature.[34] The morphological study of the CNNs was conducted by scanning electron microscopy (SEM). The SEM images reveal the layered structure of CNNs without any amorphous structure with the estimated average size of about 22 nm (Figure 1c). The zeta potential value of the 1 mgmL-1 CNNs dispersion was – 25.2 mV (Figure 1d). The presence of negative charges of the large delocalize π electron system on the CNNs provides a satisfactory dispersion of the CNNs in the matrix. This facet improves the interactions between the analyte and CNNs, and persuades high extraction efficiencies.

Amount of CNNs adsorbent

To obtain high extraction efficiencies, optimum amount of the adsorbent is essential to provide a good interaction between cyproheptadine and CNNs. For this purpose, CNNs adsorbents ranging from 10-40 mg were considered for the DSPE extraction process. As it is clear in Figure 2a, when 20 mg of the adsorbent is applied, the maximum extraction peak area is achieved. With gradual increase of the adsorbent amount to 40 mg the extraction peak area decreases. This process might be attributed to the adsorbent aggregation which cannot provide a sufficient effective surface area for cyproheptadine extraction.

Type and amount of desorption solvent

Proper desorption solvent provides maximum analyte desorption from adsorbent surface area. Therefore, kind of desorption solvent must be optimized. Three different organic solvents such as methanol, acetonitrile and acetone were considered as desorption solvents and the extraction process was carried out. The results indicated that acetonitrile provided maximum desorption peak area due to causing a sufficient interaction between the cyproheptadine and the CNNs surface (Figure 2b). The volume of the desorption solvent is of great importance in achieving a higher desorption peak area. Thus, desorption solvent volume of 200-800 µL was considered (Figure 2c). As it is clear, 200 µL of acetonitrile is sufficient to provide the maximum desorption peak area. Enhancing the volume of desorption solvent dilutes the analyte and leads in lower desorption peak areas.

Sample volume and extraction pH

Effects of sample volume on the extraction peak area was studied in the range of 3-9 mL of the sample solution. As in Figure 2d, with the gradual increase of the sample volume up to 5 mL, extraction peak area increases. Continuous increase in the sample volume diminishes the extraction peak area, which might be attributed to the weak dispersion of CNNs in the media. The Extraction pH was evaluated in the range of 6-10 (Figure 2e). As we know, π-π interactions among CNNs surface area and the cyproheptadine leads to a good adsorption of the analyte from matrix. When the pH of the media was adjusted in 6, a lower extraction peak area was obtained in account of the presence of lower negative charges in the media in acidic pH value.

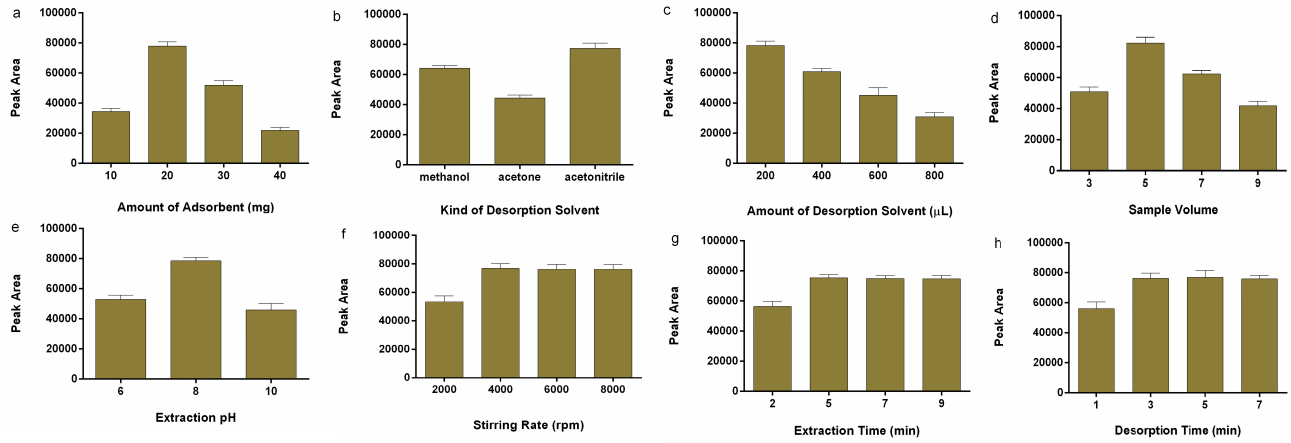

Figure 2. Optimization of the influential extraction parameters (a-h) in DSPE method in the presence of 0.5 µg/mL spiked cyproheptadine in blank sample media.

The gradual elevation of the pH to 8 resulted in a higher extraction efficiency due to the increase of the negative charges in the matrix which enhancing the cyproheptadine interaction with the CNNs. The extraction peak area decreased when the pH of the media was 10. The pKa value of cyproheptadine is 8.87 which declare that in higher pH values the analyte is in a natural form and the extraction process is just carried out due to π-π interactions, and ionic interactions in media and analyte might not be interfered.

Stirring speed, extraction and desorption time

Efficient interaction between analyte and the adsorbent is provided when analyte and the adsorbent contact thoroughly. The stirring speed might significantly affect the contact between the analyte and the adsorbent. Thus, different stirring ranges of 2000-8000 rpm were considered (Figure 2f). The results demonstrated that increasing the stirring rate up to 4000 plays a positive role in the enhancement of the extraction peak area. Gradual increase of the stirring speed up to 8000 shows no significant changes in the extraction peak area. This phenomenon proved that the system reached the equilibrium and enhancement of the stirring rate has no effect on the response of the equilibrium-based systems. The extraction time and desorption time were optimized as well (Figure 2g and h). The time range of 2-9 minutes and 1-7 minutes were considered for the extraction time and desorption time, respectively. It was observed that the extraction peak area was gained in 5 minutes at maximum, and the continuous increase of the extraction time presents no significant changes in the extraction peak area, which confirms the equilibrium establishment in 5 minutes. Besides, three minutes was the efficient time for obtaining the maximum desorption time and equilibrium establishment as well.

Method validation

Method validation was carried out by considering some figures of the merit for the novel DSPE method, and also selectivity and interference effect studies. Table 1 shows some analytical characteristics of the proposed method in cyproheptadine determination. Accuracy and repeatability of the method were investigated by inter-day and intra-day analysis of three different concentrations of spiked samples covering the calibration curve (Table 2). The results reveal that the method demonstrates satisfactory precision and accuracy. The proposed method is capable of purifying the complex matrix and avoids the impurities, like nonpolar components in the media. Based on a 95% confidence level, the intercepts of the calibration curves were not significantly different from zero. Therefore, no systematic

Table 1. Some analytical characteristics for CNNs adsorbent applied in cyproheptadine determination.

Analyte	**Concentration range** (ng/g)	**Linearity**[a] (r^2)	**LOD**[b] (ng/g)	**LOQ**[c] (ng/g)	**RSD**[d] (%)
Cyproheptadine	300-2000	0.9906	100	280	6

[a] Linearity is described by the correlation coefficient for the calibration curve; [b] Limit of Detection (LOD) S/N=3; [c] Limit of Quantification (LOQ) S/N=10; [d] Relative Standard Deviation.

Table 2. Inter-day and intra-day analysis of three different concentrations of spiked samples.

Cyproheptadine Concentration (ng/g)	**Intra-day (n=3)**		**Inter-day (n=3)**	
	Precision (RSD*)	**Accuracy** (bias)	**Precision** (RSD)	**Accuracy** (bias)
300	5.50	1.30	5.92	1.41
1000	4.87	0.98	4.98	0.96
2000	4.18	1.04	5.01	1.38

*Relative Standard Deviation

error is found for this value. The selectivity study of the present method was carried out by simultaneous spiking of 0.5 µg/mL of some other antihistamines such as chlorophenamine, diphenylhydramine and also dexamethasone as corticosteroid (Figure 3). As it is clear, there is no significant effect of other interferences in the analysis runtime of cyproheptadine. Moreover, there was no significant and well-defined peak related to the blank matrix during the analysis. The robustness of the method was studied; the results are presented in Table 3.

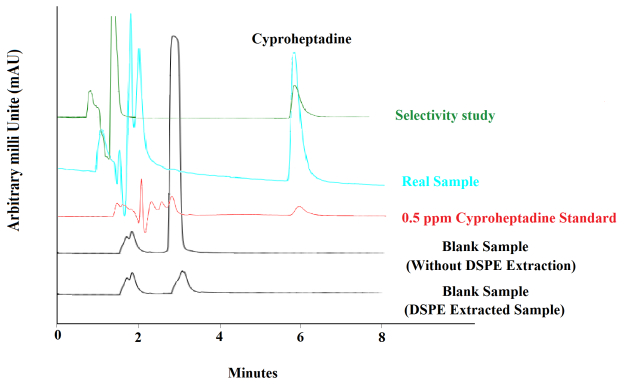

Figure 3. Chromatograms of blank sample, cyproheptadine solution, real sample and selectivity study of the introduced DSPE-HPLC-UV method in the simultaneous presence of 0.5 µg/mL cyprroheptadine, chlorophenamine, diphenylhydramine and also dexamethasone.

Table 3. Robustness study of the proposed method by one-parameter-at-a-time method.

Variable	Variations	Retention Time (min)	RSD (%)
Buffer pH	5.48	6.98	1.43
	5.5	6.20	1.28
	5.52	5.98	1.76
Desorption Solvent (µL)	210	6.40	0.8
	200	6.20	1.30
	190	6.01	1.11
Time of Extraction (min)	7	9.00	0.80
	5	6.20	0.92
	3	7.95	1.01

Analysis of real samples

Fifteen different supplement samples as tablets or capsules were provided from herbal shops and were analysed as described in the experimental section. The results are in Table 4. The mean concentration of cyprohetadine was calculated as 18.83 mg/g and the mean recovery of the extraction was 99.86%. The results confirmed the presence of cyprheptadine in plant-originated herbal supplements, which is forbidden. According to extent request of people for using herbal supplements rather than supplements containing chemical-based drugs, rigid and severe control is essential for production and distribution of these drugs in the market.

Table 4. Adulterated herbal supplements purchased from herb shops, Tabriz, Iran.

Samples Subjects	Dosage Form	Amount of Cyproheptadine (mg/g)
1	Capsules	1.68 ± 4.80
2	Capsules	2.56 ± 5.55
3	Tablet	18.90 ± 3.93
4	Tablet	22.30 ± 3.76
5	Tablet	13.70 ± 4.87
6	Capsules	5.80 ± 3.80
7	Capsules	34.65 ± 4.15
8	Capsules	29.50 ± 3.29
9	Capsules	10.59 ± 2.87
10	Capsules	19.65 ± 3.98
11	Capsules	28.84 ± 5.09
12	Tablets	40.56 ± 3.76
13	Tablets	35.78 ± 3.39
14	Tablets	14.67 ± 4.67
15	Tablets	3.67 ± 4.68

Conclusion

Great demand of the society for herbal supplements consumption highly encourages the illegal addition of chemical drugs in their content, which is not legal. The increase in children's appetite and also in adults' is an important parameter in their health, therefore, herbal supplements within cyproheptadine is highly marketed. This paper introduces a simple, fast and efficient method for extraction and determination of cyproheptadine from herbal supplements which are sold in the market. The presence of cyproheptadine in these supplements was successfully established by applying DSPE-HPLC-UV method. The results of cyproheptadine amount in several herbal samples varied in the range of 1.68-40.56 µg/g, which is much more than the amount of cyprheptadine in the commercial samples.

The results proved that severe control is of great necessity regarding the production and delivery of these synthetic and adulterant herbal products in the market.

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Conflict of Interest

The authors declare they have no conflict of interest.

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