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Biogenic Amine Determination by High-Performance Liquid Chromatography Using a Sol-Gel-Immobilized 2-Hydroxy-5-nitrobenzaldehyde-2,4-dinitro phenyl hydrazone Solid-Phase Extractant

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ABSTRACT

This study focuses on the solid phase extraction of biogenic amines (BAs) using a sol-gel adsorbent immobilized with a hydrazone ligand, named 2-hydroxy-5-nitrobenzaldehyde-2,4-dinitrophenylhydrazone. The hydrazone compound was synthesized and characterized through Fourier Transform Infrared Spectroscopy (FT-IR) and Nuclear Magnetic Resonance (NMR) spectroscopy. The efficiency of the sorbent material for extracting BAs was evaluated using the solid phase extraction (SPE) method. Key experimental parameters affecting BA extraction, including pH, equilibrium time, ligand concentration, and biogenic amine (BA) concentration, were systematically investigated. The results indicated a strong recovery of BAs from aqueous samples, demonstrating a significant affinity between the sol-gel matrix containing the hydrazone ligand and the target analytes. The findings demonstrate that incorporating the hydrazone ligand resulted in a marked enhancement of extraction efficiency at a concentation of 17×10^{-3} M. Notably, the method exhibited high selectivity for aliphatic biogenic amines such as putrescine (PUT), cadaverine (CAD), and spermidine (SPD). This extraction method was successfully applied to food samples, yielding good recovery rates.

Keywords: HPLC, SPE, extraction, Bas, Sol-Gel.

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Introduction

Biogenic amines (BAs) are low-molecular-weight nitrogenous compounds primarily formed through the decarboxylation of amino acids, as well as through amination and transamination reactions during metabolic processes. This decarboxylation removes the α-carboxyl group, yielding the corresponding amine [1]. BAs can have aliphatic, aromatic, or heterocyclic structures and are found in various food products, biological fluids, and environmental samples. The study of BAs is significant because they serve as biomarkers in toxicological risk assessments and indicators of food quality [2].

The toxicological effects of BAs vary significantly, with toxicity values largely dependent on the efficiency of detoxification mechanisms for each compound. Food products that undergo natural fermentation, such as cheese, soybean products, and alcoholic beverages, are particularly susceptible to BAs due to the presence of contaminating microflora that exhibit amino acid decarboxylase activity [3]. Numerous studies have reported the presence of BAs in various contexts, including human tissues [3], plants [4], and food products like fish and seafood [[5], [6]], meat products [[7], [8]], bean products [9], fruits and vegetables [[10], [11]], and beverages [[12], [13]]. Notable biogenic amines, such as histamine, tyramine, putrescine, cadaverine, spermidine, and spermine, are significant due to their physiological activity and potential adverse health effects at high concentrations [14]. Therefore, accurately determining the levels of

biogenic amines is crucial for food quality control, clinical analysis, and environmental monitoring.

Several analytical methods, including liquid-liquid extraction (LLE), liquid-phase microextraction (LPME), solid-phase extraction (SPE) [15], and solid-phase microextraction (SPME) [16], as well as techniques such as spectrophotometry [17], capillary electrophoresis [18], gas chromatography [19], and high-performance liquid chromatography (HPLC) [20], have been developed for this purpose. Among these, HPLC is particularly favored for its high sensitivity, selectivity, and compatibility with a wide range of analytes. However, directly measuring biogenic amines can be challenging due to their high polarity, lack of strong chromophores, and low concentrations in complex matrices [21]. Therefore, an effective sample preparation step is often necessary to enhance detection limits and minimize matrix interferences. The SPE method has emerged as a widely used sample preparation technique due to its simplicity, high enrichment capability, and low solvent consumption. The effectiveness of this method largely depends on the type of sorbent material used [22]. Recently, the development of selective and chemically modified sorbents has garnered significant attention. Notably, sol–gel technology offers considerable advantages for sorbent preparation, including high chemical and thermal stability, controllable porosity, and the ability to immobilize functional organic ligands within an inorganic framework [[23], [24]]. This study aimed to extract biogenic amines (BAs) using a solid-phase method with a sol-gel adsorbent that had been modified with the hydrazone ligand 2-hydroxy-5-nitrobenzaldehyde-2,4-dinitro phenyl hydrazone. The amounts of these amines were then measured with high-performance liquid chromatography (HPLC).

Experimental part

Materials

2,4-Dinitrophenylhydrazine (DNPH) (Aldrich), 2-Hydroxy-5-nitrobenzaldehyde (Fluka), cadaverine (CAD) dihydrochloride, histamine (HIS) dihydrochloride, putrescine (PUT) dihydrochloride, β-phenylethylamine (PEA) dihydrochloride, spermidine (SPD) trihydrochloride, tryptamine (TRY) hydrochloride, and tyramine (TYR) hydrochloride were all obtained from Sigma-Aldrich. Dansyl chloride (Dns-Cl) (Fluka), hydrochloric acid (Across), L-glutamic acid monosodium monohydrate (Across), sodium hydroxide (Across), sodium hydrogen carbonate (Across), sulfuric acid (Across), tetraethoxysilane (TEOS) (Sigma-Aldrich), and tris(hydroxymethyl)aminomethane (Tris) (Sigma-Aldrich) were also used. All chemicals and reagents were used as received without further purification.

Instruments

Elemental analysis (CHN) of the ligand and its sorbent material were carried out using a Perkin–Elmer 240011 elemental analyzer. Infrared analysis was performed on a Perkin–Elmer 2000 FT–IR unit using a KBr system. The structure of the ligand was further confirmed by H NMR using a Bruker 400 MHz spectroscopy. Thermogravimetric analyses (TGA) were carried out using a Perkin–Elmer Thermo Gravimetric Analyzer TGA 7 under nitrogen gas, scanning rate of 10 °C/min. A Perkin-Elmer Lambda 35 (dual beam) spectrophotometer was used to record the spectra of the ligand and sorbent material. A Perkin Elmer 200-series HPLC unit consisting of a pump, vacuum degasser, auto sampler, diode array detector, and C_{18} ODS Hypersil column (250 × 4.5 mm, 5 µm) was used. The mobile phase was acetonitrile: water: methanol: (60: 25: 15) at a flow rate of 1.0 mL min^{-1}.

Synthesis of 2-hydroxy-5-nitrobenzaldehyde-2,4-dinitrophenylhydrazone

The ligand was synthesized as previously reported by Tameem et al. [25]. A solution containing 0.0057 M (1.6 g) of DNPH in 30 mL of ethanol was thoroughly mixed with 0.0081 M (1.35 g) of 2-hydroxy-5-nitrobenzaldehyde in 10 mL of ethanol. An orange solid precipitate formed, which was then filtered and dried overnight in an oven at 70 °C. The yield was 2.65 g (94%), with a melting point of 300 °C (Figure 1).

Preparation of the sorbent

The sorbent was prepared as previously reported [25]. The ligand was physically immobilized in the silica sol–gel matrix by stirring a mixture of TEOS (3.28 mL), ethanol (4.56 mL), and HCl (0.36 mL, 4 M) in a 50 mL beaker for 15 minutes to create a sol solution. An appropriate amount (0.017 g) of the hydrazone ligand (L), dissolved in 10 mL of THF, was added to the sol solution and stirred vigorously for 45 minutes. After homogenization, the mixture was aged in an oven at 60 ºC for 2 days. The sol-gel product was then soaked in water for 1 day for conditioning and subsequently dried at 60 ºC. A blank sorbent was prepared similarly, without the ligand.

Extraction of biogenic amine

The extraction process of BAs was conducted according to our previously reported method [[25], [26]] using a batch extraction technique. A 25 mg sorbent was placed in a glass vial with 1 mL of a BAs standard mixture (100 mg L-1) and 4 mL of 0.1 M Tris buffer (pH 8-10). The vial was then shaken mechanically at room temperature for 30 minutes. After the equilibrium period, the mixture was processed as previously described [25]. The percentage of extraction was calculated using the following equation:

$$\%E = \frac{C_0 - C}{C_0} \times 100$$

where, C_0 and C are the BA concentration (mg. L^{-1}) in the solution before and after extraction, respectively.

Results and discussion

Extraction of biogenic amine

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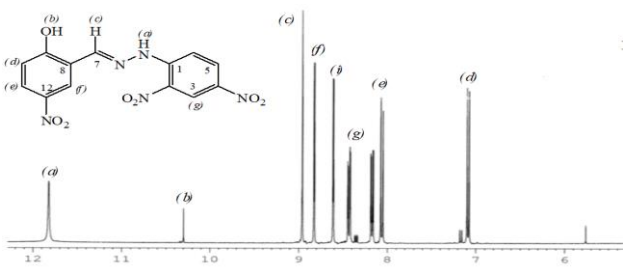

Figure 1 - HNMR of 2-Hydroxy-5-nitrobenzaldehyde-2,4-dinitrophenylhydrazone

Characterization of sol-gel sorbent

Solid UV-Vis analysis

The FTIR analysis did not provide conclusive evidence of hydrazones in the gel matrix, as the spectra of the blank and the sorbent were too similar. This lack of distinction is likely due to the very small amounts of ligand present in the network, a challenge noted by other researchers [[27], [28]]. Subsequently, solid-state UV-Visible analysis was conducted. A comparison was made between the free ligand and its corresponding sol-gel sorbent using solid-state UV-Vis spectroscopy in the 200 to 800 nm range. The free ligand exhibited a maximum absorption (λ_{max}) at 382 nm, which displayed a red shift compared to the sol-gel sorbent, which had a λmax of 381 nm (Figure 2). The notable difference between the spectra of the blank and the sorbent provides strong evidence for the successful incorporation of the hydrazone ligand into the sol-gel network. This slight shift may be attributed to intermolecular interactions between the ligand and silica, which could alter the characteristics of the absorption spectra [[27], [28]]

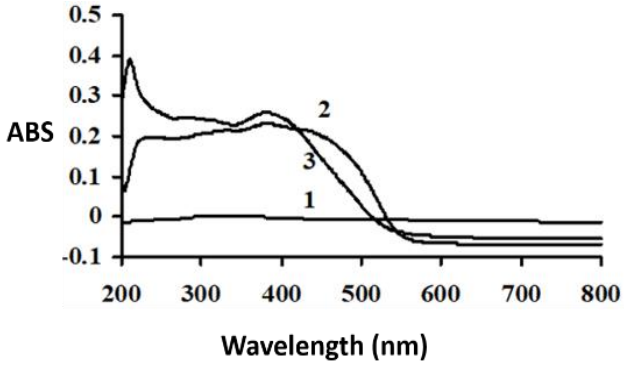

Figure 2 - UV-Vis spectra of (1) the blank sol-gel sorbent, (2) free ligand (2) and (3) sol-gel sorbent

HPLC conditions

An earlier HPLC method [25] was utilized to separate BA using a three-solvent mobile phase mixture: methanol (MeOH) (0.0 - 20%), water (20 - 40%), and acetonitrile (ACN) (50 - 70%). The flow rate was maintained between 0.8 and 1.4 mL min^{-1} at ambient temperature. The optimal separation occurred at a flow rate of 1.0 mL min^{-1} with a mobile phase composition of 60:25:15 (v/v/v) (ACN:H_2O:MeOH). Under these conditions, dansylated amines were detected at 254 nm, and all components of BA were eluted in under 18 minutes. To assess the linearity of the response, a standard mixture of BA was prepared, ranging from 0.001 to

50 mg L^{-1}, and injected. Calibration graphs for each BA were created by plotting the peak area against concentration

Optimized parameters for extraction

The effect of pH

The study examined the impact of sample pH on extraction efficiency. It was determined that the optimal pH range for the quantitative extraction of BA lies between 8 and 11 (Figure 3). Within this range, the sorbent achieved extraction efficiencies close to 100%, particularly for SPD, followed by CAD and His. Deprotonation occurs at the N-H amino group [[26], [29]], enhancing delocalization resonance and creating additional charge interactions between the protic BA and the N-H group. This finding underscores the significant role of ligand polarity in the interaction between the studied sorbent material and amines within this pH range [26]. Conversely, the low extraction efficiency observed in acidic conditions may result from the high ionization potential of BA at lower pH levels or repulsion between the protic BA and the protic (N-H) group on the ligand. Additionally, the sorbent material contains a ligand with an extra polar OH group, which may enhance electrostatic interactions with BA.

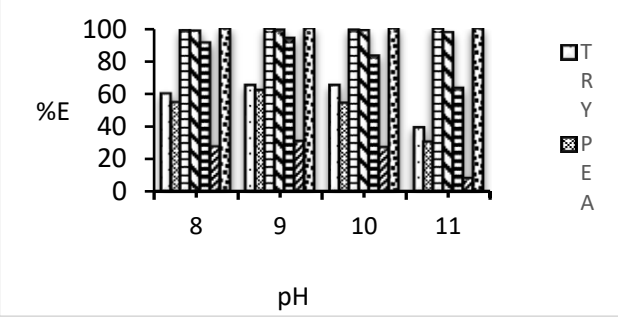

Figure 3 - Effect of pH on the extraction of BA (20 mg L^{-1}).

Effect of contact time

The effect of contact time on the extraction efficiency was studied by shaking the BA mixture with the adsorbent, ranging from 5 to 60 min at optimum pH 9. Good extraction (%E > 70 %) was observed after 5 min of contact time for all studied amines (Figure 4). The process was maximized after 30 min of contact time, especially for SPD, CAD, and PUT. No significant effect on extraction was observed after this. Therefore, 30 min contact time was chosen for subsequent studies [30].

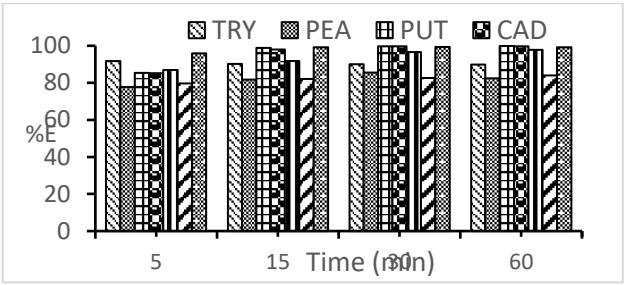

Figure 4 - Effect of contact time on the extraction of BA (20 mg L^{-1}); pH 9.

Effect of ligand concentration in the sol - gel network

The effect of the concentration of the immobilized ligand on the extraction efficiency of BAs was also examined. A 25 mg sample of the adsorbent with varying ligand concentrations ranging from 8.5 to 68 × 10^{-3} M was used to extract 20 mg L^{-1} BAs from an aqueous solution at the optimal pH of 9. More than 80% of BA was extracted when 17 × 10^{-3} M of the ligand was used (Figure 5). Increasing the ligand concentration beyond this point did not further enhance extraction efficiency. In contrast, no extraction was observed in the absence of the ligand (Blank sol-gel). These results indicate that the addition of the hydrazone ligand significantly improved extraction efficiency [31].

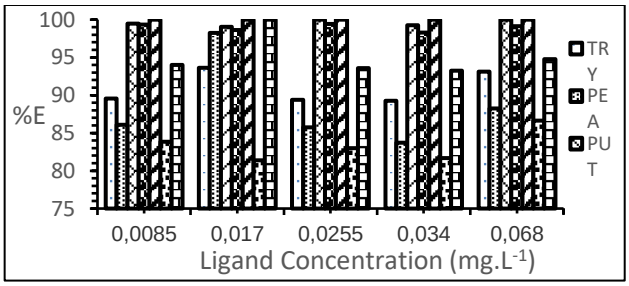

Figure 5 - Effect of ligand concentration on the extraction of BA, pH 9, contact time 30 min.

Effect of biogenic amine

Five different concentrations (5.0-500 mg L^{-1}) of BAs were used to evaluate the extraction efficiency and capacity of the sorbent. It was found that at lower concentrations (5 mg L^{-1}), most of the studied BAs, including SPD, TRY, P

UT, CAD, and HIS were extracted efficiently (100% extraction) (Figure 6). The sorbent demonstrated high extraction efficiency for SPD even at 500 mg L-1. However, a decrease in extraction efficiency was observed at concentrations higher than 200 mg L^{-1}. Among the BAs, the aromatic

compounds (HIS, TYR, PEA) were extracted the least effectively within this concentration range [33].

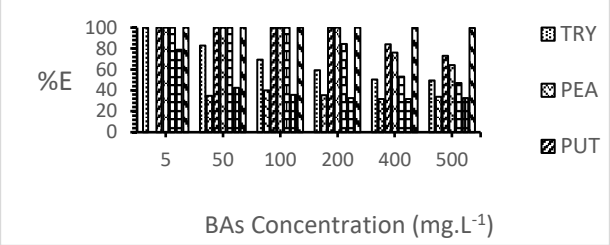

Figure 6 - Effect of biogenic amine (BA) concentration on the extraction.

Capacity of sorbents

The capacity of the sorbent material was found to be dependent on the initial concentration of these analytes in the solution (Figure 7). As the concentration of SPD, PUT, CAD, and HIS increase, the capacity value of the sorbent also increases (PUT > HIS > SPD > CAD). The highest value of the capacity was found for PUT (0.0023 - 0.220 mmol g^{-1}). The capacity of this sorbent is slightly inferior when compared to the sorbents based on silica gel immobilized aliphatic amines and imprinted organic – inorganic hybrid (e.g., 0.1 - 3.1 mmol g^{-1}) but higher than the sorbents based on the immobilized ligands such as crown ethers (0.0016 – 033 and 0.0038 – 0.00086 mmol g^{-1}) [[34], [35]].

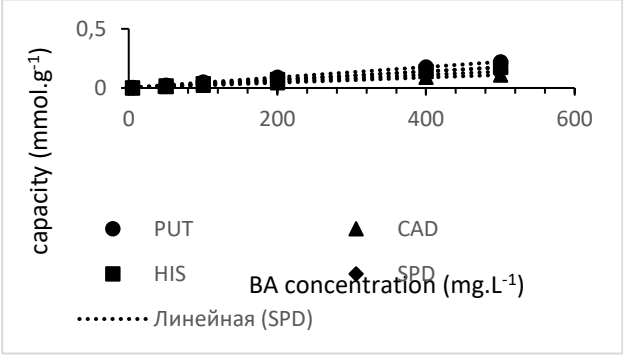

Figure 7 - The capacity of sorbent (mmol g^{-1}) with different concentrations of BA.

Adopted extraction conditions

The adopted extraction conditions were: pH, 9; contact time, 30 min; ligand concentration, 17×10^{-3} M; sorbent mass, 25 mg; and room temperature, 25 °C.

Extraction of BA from food samples

The proposed method was applied for the extraction and determination of biogenic amines (BAs) in ketchup. Table 1 presents the recovery results. Representative chromatograms of the unspiked sample extract (Figure 8) and the spiked sample (5 mg L^{-1} BA) before and after extraction (Figure 9) are included. The effectiveness of the sorbent in removing BA from the sample matrices is evident. All food samples achieved satisfactory recoveries of over 85% for the spiked 5 mg L^{-1} BA. Additionally, good recoveries were observed for putrescine (PUT), cadaverine (CAD), and spermidine (SPD). These high recovery rates for PUT, CAD, and SPD from food matrices suggest that this method can be successfully applied for determining these BAs in various foods [[36], [37], [38]].

Table 1 - Recoveries (%) of BA from spiked (5 mg L^{-1} BA) ketchup food sample.

	BA						
	TRY	PEA	PUT	CAD	HIS	TYR	SPD
%E	93.7	85.6	100	100	90.1	88.0	99.0
±SD	1.9	2.0	1.3	0.1	0.3	0.8	0.0

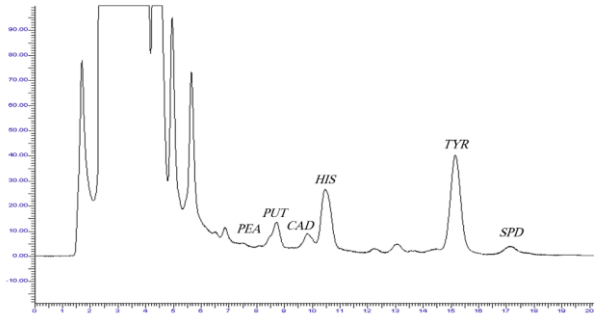

Figure 8 - Chromatogram of ketchup extract. Peak identification.

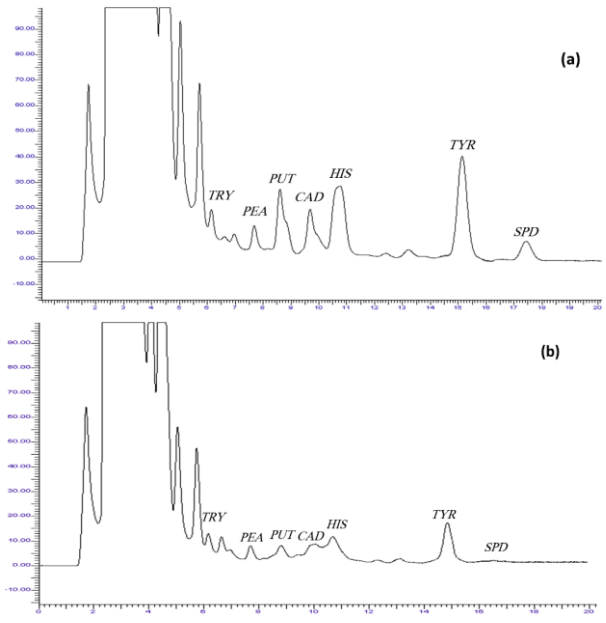

Figure 9 - Chromatogram of ketchup extract, spiked with 5 mg L^{-1} BA, (a) Before extraction and (b) after extraction.

Conclusion

The hydrazone ligand, synthesized and immobilized within a sol-gel matrix, was evaluated for its performance in solid-phase extraction (SPE) of biogenic amines (BAs). Derived from 2-hydroxy-5-nitrobenzaldehyde-2,4-dinitrophenylhydrazone, this sorbent demonstrated marked selectivity towards aliphatic biogenic amines, including putrescine (PUT), cadaverine (CAD), and spermidine (SPD). The enhanced extraction efficiency stems from the strong interaction between the hydrazone-containing sol-gel matrix and the target analytes. This approach proved effective for extracting biogenic amines from ketchup samples. Furthermore, hydrazone compounds offer a significant advantage over crown ether-based sorbents by enabling straightforward single-step synthesis at ambient temperature [[25], [38]].

Conflicts of interest. Authors declare no conflict of interest.

CRediT author statement: **A.A. Tameem:** Conceptualization, Methodology, Software. **S.S. Mohamed**: Data curation, Writing draft preparation. **A.S. Alnaas**: Visualization, Investigation. **E. Kusrini**: Reviewing and Editing

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Иммобилизацияланған 2 гидрокси-5 нитробензальдегид 2,4-динитрофенилгидразонды қатты фазалы экстрагентті золь гелін қолдана отырып жоғары өнімді сұйық хроматография арқылы биогенді аминдерді анықтау

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ТҮЙІНДЕМЕ

Бұл зерттеу иммобилизацияланған 2-гидрокси-5-нитробензальдегид-2,4-динитрофенилгидразон деген атауы бар гидразон лигандымен золь-гель адсорбентін қолданып биогенді аминдерді (БА) қатты фазалы экстракциялауға арналған . Гидразон қосылысы ИК және ЯМР спектроскопиясы арқылы синтезделіп, сипатталды. Қатты фазалы экстракциялау (ҚФЭ) әдісін қолдану арқылы биогенді аминдердің экстракциясына сорбциалық материалдың тиімділігі бағаланды . Биогенді аминдердің экстракциясына әсер ететін негізгі эксперименттік параметрлер: соның ішінде pH, тепе-теңдік уақыты, лиганд концентрациясы және ҚФЭ концентрациясы жүйелі түрде зерттелді. Нәтижелер сулы үлгілерден биогенді аминдердің жоғары дәрежеде қалпына келтірілгенін көрсетті, бұл гидразон лигандтары бар золь-гель матрицасы мен нысаналы аналиттер арасындағы айтарлықтай жақындықты көрсетті. Айта кетсек бұл әдіс путресцин (PUT), кадаврин (CAD) және спермидин (SPD) сияқты алифатты биогенді аминдерге жоғары селективтілік көрсетті. Жақсы экстракция көрсеткіштерін қамтамасыз ету арқылы бұл әдісі тағам үлгілеріне сәтті қолданылды.

Түйін сөздер: ЖӨСХ, ҚФЭ, экстракция, биогенді аминдер , золь -гель.

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Определение биогенных аминов методом высокоэффективной жидкостной хроматографии с использованием твердофазного экстрагента на основе золь-геля, иммобилизованного на 2-гидрокси-5-нитробензальдегиде и 2,4-динитрофенилгидразоне

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АННОТАЦИЯ

Данное исследование посвящено твердофазной экстракции биогенных аминов (БА) с использованием золь-гелевого адсорбента, иммобилизованного гидразоновым лигандом, названным 2-гидрокси-5-нитробензальдегид-2,4-динитрофенилгидразоном. Гидразоновое соединение было синтезировано и охарактеризовано с помощью ИК-спектроскопии и ЯМР-спектроскопии. Эффективность сорбционного материала для экстракции биогенных аминов оценивалась с использованием метода твердофазной экстракции (ТФЭ). Были систематически исследованы ключевые экспериментальные параметры, влияющие на экстракцию биогенных аминов , включая pH, время достижения равновесия, концентрацию лиганда и концентрацию биогенных аминов. Результаты показали высокую степень извлечения биогенных аминов из водных образцов, демонстрируя значительное сродство между золь-гелевой матрицей, содержащей гидразоновый лиганд, и целевыми аналитами. Примечательно, что метод показал высокую селективность по отношению к алифатическим биогенным аминам, таким как путресцин (PUT), кадаверин (CAD) и спермидин (SPD). Данный метод экстракции был успешно применен к образцам пищевых продуктов, обеспечив хорошие показатели извлечения.

Ключевые слова: ВЭЖХ, ТФЭ, экстракция, биогенные амины, золь-гель.

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