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Ultrasound-assisted supramolecular deep eutectic solvent extraction coupled with salting-out separation for the determination of GABA in tea

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Research Article

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Ultrasound-assisted supramolecular deep eutectic solvent extraction coupled with salting-out separation for the determination of GABA in tea

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Ultrasound-assisted supramolecular deep eutectic solvent extraction coupled with salting-out separation for the determination of GABA in tea

Abstract

In this work, a convenient, sensitive, and economical ultrasound assisted extraction method based on a supramolecular deep eutectic solvent (SUPRADES) was developed for the determination of γ-aminobutyric acid (GABA) in tea. In this approach, 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CD) served as the hydrogen-bond acceptor and malonic acid as the hydrogen-bond donor for the preparation of the DES, while ultrasound assistance was employed to intensify mass transfer. The DES composition and key process variables were systematically investigated and optimized. The effects of DES dosage, ultrasonication time, solid-to-liquid ratio, salt amount, and derivatization time were evaluated with a Box–Behnken response surface. The obtained model was highly significant ($p < 0.0001$) with an not significant lack of fit ($p = 0.0853$), affording an R^2 of 0.9995. Under the optimized conditions, excellent linearity was achieved over 0.5–500 μg/mL (R^2 = 0.9995), with a limit of detection (LOD) and limit of quantification (LOQ) of 0.27 μg/mL and 1.25 μg/mL, respectively. The method exhibited satisfactory intra /inter-day precision (RSD ⩽ 2.7%) and recoveries of 85.6%–92.8%. The greenness of the developed method was assessed using two quantitative green metrics tools: analytical Eco-scale and AGREE, with analytical Eco-scale qualifying the proposed method as green. The proposed method was successfully applied to real tea samples and exhibited a limited matrix effect, offering a practical sample-preparation strategy for other complex food matrices.

Keywords:γ-aminobutyric acid; supramolecular deep eutectic solvent; ultrasound assisted extraction; Eco-scale; AGREE

Introduction

Tea is a widely consumed botanical beverage and a rich source of low-molecular-weight bioactive compounds. Among them, γ-aminobutyric acid (GABA) is a representative non-proteinogenic amino acid[1,2]. In addition to contributing to taste, GABA is a neuroactive molecule that has been associated with the regulation of blood pressure and sleep[3]. With growing consumer interest in "GABA-rich" specialty teas, reliable quantitative methods are needed to support quality control and substantiate functional claims. Accurate measurement, however, is complicated by the highly complex tea matrix, which contains abundant polyphenols, caffeine, and pigments that can interfere with the selective isolation and determination of GABA[4]. Most quantitative methods for γ-aminobutyric acid (GABA) rely on liquid chromatography (LC) or capillary electrophoresis (CE)[5,6]. Because GABA has little intrinsic UV/visible absorbance, pre-column or on-column

derivatization is usually required to achieve sufficient detection[7]. In practice, however, overall performance is often limited by sample pretreatment, which is commonly regarded as the main bottleneck of the workflow. Conventional extraction procedures (e.g., refluxing or maceration) can be time-consuming and lack selectivity, leading to co-extraction of matrix components[8,9]. Such coextractives may elevate baseline noise and suppress the analytical signal, thereby compromising quantification[10].

To address these issues, deep eutectic solvents (DESs) have been increasingly explored for sample preparation. Over the past decade, DESs have gained broad interest because they are generally less toxic and more biodegradable than many conventional organic solvents. A key advantage of DESs is their tunability, where the selection of different hydrogen-bond donors (HBDs) and acceptors (HBAs) allows for the precise tailoring of their polarity, viscosity, and extraction behavior to the target analyte and matrix[11-13]. The conventional preparation of DESs generally involves the prior mixing of HBD and HBA at a predetermined ratio[14]. This mixture is subsequently subjected to heating and stirring until a homogeneous and transparent liquid is formed, which is then employed for sample extraction. Recent studies have led to the emergence of a new subclass of deep eutectic solvents, namely supramolecular deep eutectic solvents (SUPRADESs).

Cyclodextrin (CD), a natural cyclic oligosaccharide, can serve as the hydrogen bond acceptor in SUPRADESs. Owing to its characteristic truncated-cone structure, CD possesses a hydrophilic exterior and a hydrophobic inner cavity[15]. SUPRADESs not only retain the environmentally benign features and physicochemical characteristics of conventional DESs, but also exhibit selective binding ability toward a wide range of compounds because of their supramolecular nature. In many reported applications, SUPRADESs have shown promising or improved performance in selected applications. By combining the green attributes of DESs with the distinctive structural features of cyclodextrins, SUPRADESs provide a unique host and guest interaction mechanism that is expected to improve the extraction of target analytes[16].For instance, Huang et al. [17] developed a cyclodextrin-based supramolecular deep eutectic solvent (SUPRADES) for the ultrasonic-assisted extraction of tea polyphenols, demonstrating its potential as a green medium for recovering plant-derived bioactive compounds. Similarly, Pietrangeli et al. [18] designed a β-cyclodextrin-containing SupraDES system for the extraction of phenolic compounds from olive pomace, further confirming the applicability of SUPRADESs in the valorization of agro-industrial byproducts. In another representative study, Zhang et al. [19] reported a pH-adjustable ternary cyclodextrin-based SUPRADES for the efficient extraction and analysis of trace quinolones and sulfonamides in wastewater, highlighting its suitability for the selective enrichment of contaminants from complex aqueous matrices. More recently, Sadek et al. [20] established a SUPRADES-based dispersive liquid–liquid microextraction method coupled with HPLC for the determination of melamine in milk samples, demonstrating the feasibility of this solvent system for food analysis. Collectively, these studies suggest that SUPRADESs can integrate the tunable physicochemical properties of

deep eutectic solvents with the host–guest recognition capability of cyclodextrins, thereby offering promising extraction efficiency and selectivity across diverse food and environmental matrices.

In this study, we developed an ultrasound-assisted extraction strategy based on a SUPRADES combined with salting-out assisted liquid-liquid microextraction (SALLME). The SUPRADES was prepared using 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CD) as the hydrogen bond acceptor and malonic acid as the hydrogen bond donor. Comprehensive investigations were carried out to evaluate the effects of key parameters, including the type of extraction medium, ultrasonication time, and solid-to-liquid ratio, on the enrichment efficiency of GABA from tea samples. In addition, the sustainability and environmental performance of the proposed method were systematically assessed using greenness evaluation tools, namely the Analytical Eco-Scale (AES) and the Analytical GREEnness metric (AGREE) [21].

Materials and Methods

Chemicals and materials

Tea samples were purchased from the Taobao online marketplace (China). GABA reference standard (purity 99.9% or higher) was obtained from Sigma (USA). 4-dimethylaminoazobenzene-4-sulfonyl chloride, 2-hydroxy-beta-cyclodextrin, sodium bicarbonate, malonic acid, DL-lactic acid, citric acid, formic acid, and dimethyl phosphate (analytical grade) were purchased from Macklin (China). Acetonitrile and acetic acid (HPLC grade) were supplied by Sigma (USA). Wahaha ultrapure water was used throughout the experiments.

Instruments

Chromatographic analysis was performed using a Waters e2695 HPLC system (Waters, Milford, MA, USA). Thermogravimetric analysis (TGA) was conducted on a Hitachi STA200 simultaneous thermal analyzer (Hitachi, Japan). Fourier transform infrared (FT-IR) spectra were recorded using a Nolay-50 FT-IR spectrometer (Tianjin Nolay Scientific Instruments Co., Ltd., China). The mass of the samples was measured using an ML204 analytical balance (Mettler-Toledo, USA).

Software

Data processing was performed using Excel 2016, while all graphical representations including FT-IR spectra, TGA curves, and HPLC chromatograms were generated using Origin 2025b. The optimization of key experimental parameters, such as amount of DES, ultrasonication time and so on, was conducted through Response Surface Methodology (RSM) based on a Box-Behnken Design (BBD) using Design-Expert software. Furthermore, the environmental impact of the developed method was evaluated using AGREE software for overall greenness assessment, and the Analytical Eco-Scale (AES) was employed to assess its environmental and practical sustainability.

HPLC-UV conditions

All samples were analyzed on a Waters e2695 HPLC system equipped with an autosampler and a UV-Vis detector.The detection wavelength was set to 436 nm. Separation was performed on a C18 column (5 μm, 4.6 × 250 mm) under isocratic elution at 30 °C. The injection volume was 2 μL. Mobile phase A was acetonitrile, and mobile phase B was a sodium acetate solution (20:80, v/v). The flow rate was maintained at 1.0 mL/min, and the total run time for each sample was 15 min.

Extraction and Derivatization of GABA

Preparation of Deep Eutectic Solvents

DESs were prepared by mixing an HBA and an HBD at predetermined mass ratios, followed by heating in a water bath at 80 °C until a homogeneous phase was obtained. Four DES systems with different HBA and HBD combinations were evaluated in this study, and their corresponding compositions and mass ratios are summarized in Table 1.

Table1 Different systems of DESs.

Abbreviation	HBA	HBD	Mass ratio
DES-1	2-HP-β-CD	DL-lactic acid	1:1;1:2;1:3;1:4
DES-2	2-HP-β-CD	Malonic acid	1:1;1:2;1:3;1:4
DES-3	Glycerol	Malonic acid	1:0.5;1:1.1;1:1.6;1:2.2
DES-4	Propylene glycol	Malonic acid	1:1;1:2;1:3;1:4

Extraction and derivatization procedure

Tea samples were ground to a homogeneous powder, 2.0 g of the powder was accurately weighed into a 10 mL centrifuge tube, followed by the addition of 5 mL deionized water and 1.0 mL DES. The mixture was subjected to ultrasonic extraction for 30 min. The resulting homogenate was then centrifuged at 5000 rpm for 5 min, and the supernatant was transferred to a new centrifuge tube. K_2HPO_4 was added to the supernatant at the specified amount, and the solution was vortex-mixed for 5 min until the salt completely dissolved. The mixture was subsequently centrifuged again at 5000 rpm for 5 min to obtain a clear phase separation, with GABA enriched in the upper phase. The upper enriched phase was collected and diluted fivefold with deionized water to reduce viscosity and salt loading. The diluted extract was purified using a C18 solid phase extraction cartridge to remove interferences from tea polyphenols and pigments. The cartridge was sequentially conditioned and equilibrated with methanol and deionized water, respectively, before sample loading, and the eluate was collected for derivatization. Specifically, a 1.0 mL aliquot of the eluate was mixed with 0.2 mL sodium bicarbonate solution and 0.4 mL of the 4-dimethylaminoazobenzene-4-sulfonyl chloride derivatization reagent. After thorough mixing, the reaction was carried out in a 70 °C water bath for 20 min. The derivatized solution was filtered through a 0.45 μm membrane filter prior to HPLC analysis. A schematic illustration of the workflow is shown in Fig. 1.

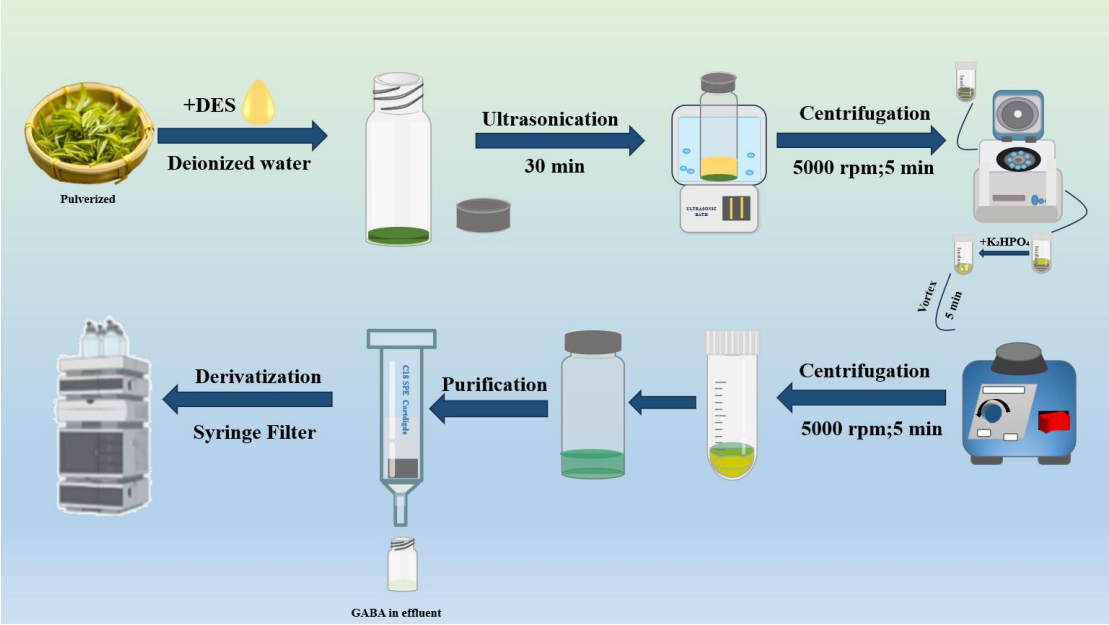


Fig. 1 Schematic illustration of the extraction procedure.

Experimental design and statistical evaluation

To elucidate the interactions among the five independent variables and to determine the optimal extraction conditions, a Box-Behnken design (BBD) with five factors at three levels was employed, resulting in 46 experimental runs. Quadratic polynomial regression and analysis of variance (ANOVA) were performed using Design-Expert software (version 13). The statistical significance of the obtained model was evaluated through ANOVA based on the p-value, the coefficient of determination (R^2), the adjusted R^2_{adj}, and the predicted $R^2_{pred.}$

Methods of validation and calculating formulas

The recovery percentage of the developed method was determined based on the parameters associated with Eq. (1).

$$(1)\ RE\% = \frac{C_{spiked} - C_0}{C_{add}} \times 100\%$$

C_0 denotes the concentration of the analyte in the unspiked sample, whereas C_{spiked} represents the concentration quantified in the spiked sample.C_{add} refers to the nominal concentration added to the sample during the spiking process.

The sensitivity of the developed method was evaluated by determining the limit of detection (LOD) and the limit of quantification (LOQ). According to the recommendations of IUPAC and AOAC, LOD is defined as the lowest concentration of an analyte that can be reliably detected but not necessarily quantified, whereas LOQ represents the lowest concentration that can be determined with acceptable accuracy

and precision. The mathematical expressions for these parameters are given in Eq (2) and (3):

$$(2)\ \mathrm{LOD}=\frac{3.3\sigma}{S}$$

$$(3)\ \mathrm{LOQ}=\frac{10\sigma}{S}$$

S represents the slope of the calibration curve, and σ denotes the residual standard deviation of the response, which was determined using the ANOVA statistical tool. The coefficients 3.3 and 10 are based on the required signal-to-noise (S/N) ratios for detection and quantification, respectively.The precision of the developed method was expressed as the relative standard deviation (RSD, %), which was calculated according to Eq (4):

$$(4)\ \mathrm{RSD}=\frac{SD}{\bar{C}}\times 100\%$$

In this equation, SD denotes the standard deviation, while $\bar{c}$ represents the mean concentration of the replicates.

To evaluate the matrix effect (ME) on the GABA analysis, the slopes of the matrix-matched calibration curve and the solvent-based calibration curve were compared according to the following Eq(5):

$$(5)\ \mathrm{ME}=\frac{S_{matrix}}{S_{solvent}}\times 100\%$$

Where S_{matrix} and $S_{solvent}$ represent the slopes of the calibration curves obtained from the matrix-matched standards and solvent-based standards, respectively.

FT-IR analysis

FT-IR spectroscopy was employed to confirm the successful formation of the DES. The FT-IR results revealed an evident red shift of the O-H stretching band of 2-HP-β-CD in the 3600 to 3000 cm minus one region, indicating the establishment of intermolecular hydrogen bonding. In addition, band broadening was observed, further supporting the presence of extensive hydrogen-bond interactions within the synthesized DES[22,23]. Fig. 2 compares the FT-IR spectra of the DES with those of the individual components, namely 2-hydroxypropyl-beta-cyclodextrin and malonic acid. The band at 1649 cm^{-1}, attributed to malonic acid, increased markedly in intensity in the DES spectrum. Moreover, characteristic bands of 2-HP-β-CD at 2929, 1458, and 1381 cm^{-1} shifted to 2917, 1438, and 1404 cm^{-1}, respectively, accompanied by notable intensity variations. Collectively, these spectral changes confirm the formation of strong hydrogen-bond interactions between the two

components, supporting successful DES formation.

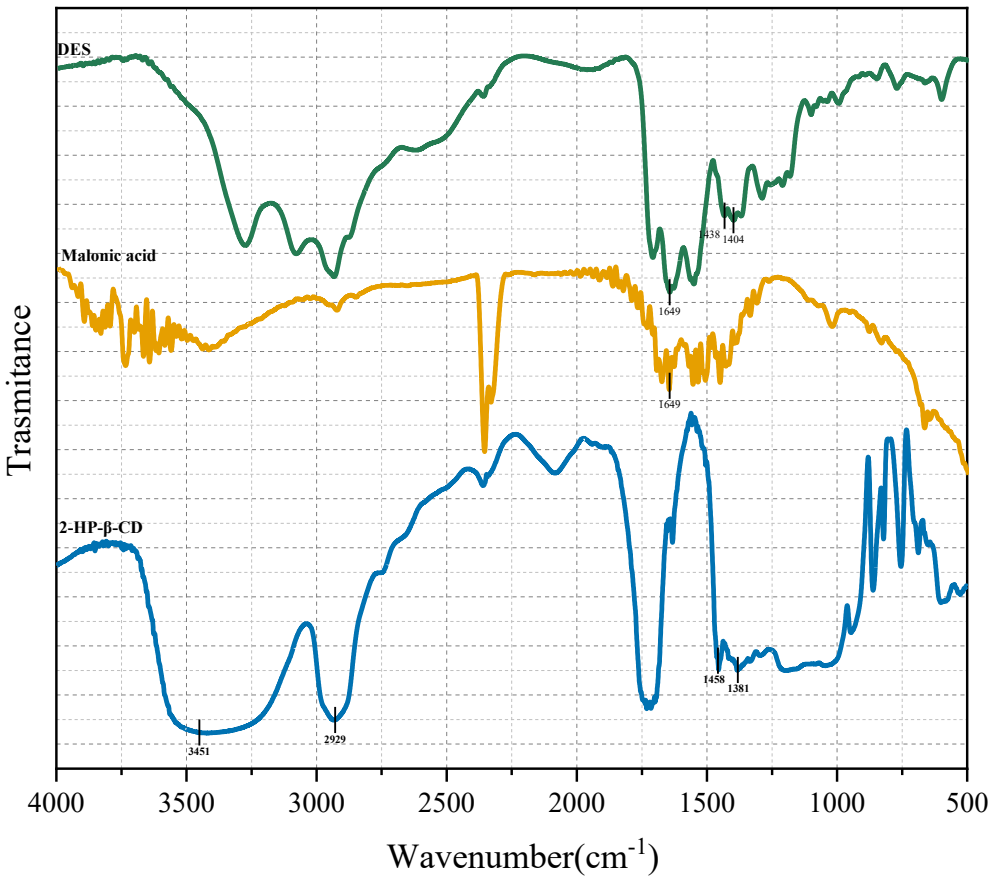


Fig. 2 FTIR spectra of 2-HP-β-CD, Malonic acid and DES.

Thermogravimetric analysis

To assess the thermal stability of the prepared DES, thermogravimetric (TG) measurements were performed for the DES and its individual components, 2-HP-β-CD and malonic acid. The results are shown in Fig. 3. At the end of the heating program, all three samples exhibited nearly zero residual mass, indicating that they underwent almost complete thermal decomposition at elevated temperature and that inorganic residues were negligible. Compared with the two pure components, the onset temperature of the main thermal decomposition step for the DES increased to 195 °C, demonstrating improved thermal stability and a higher permissible operating temperature, which is advantageous for extraction related applications.

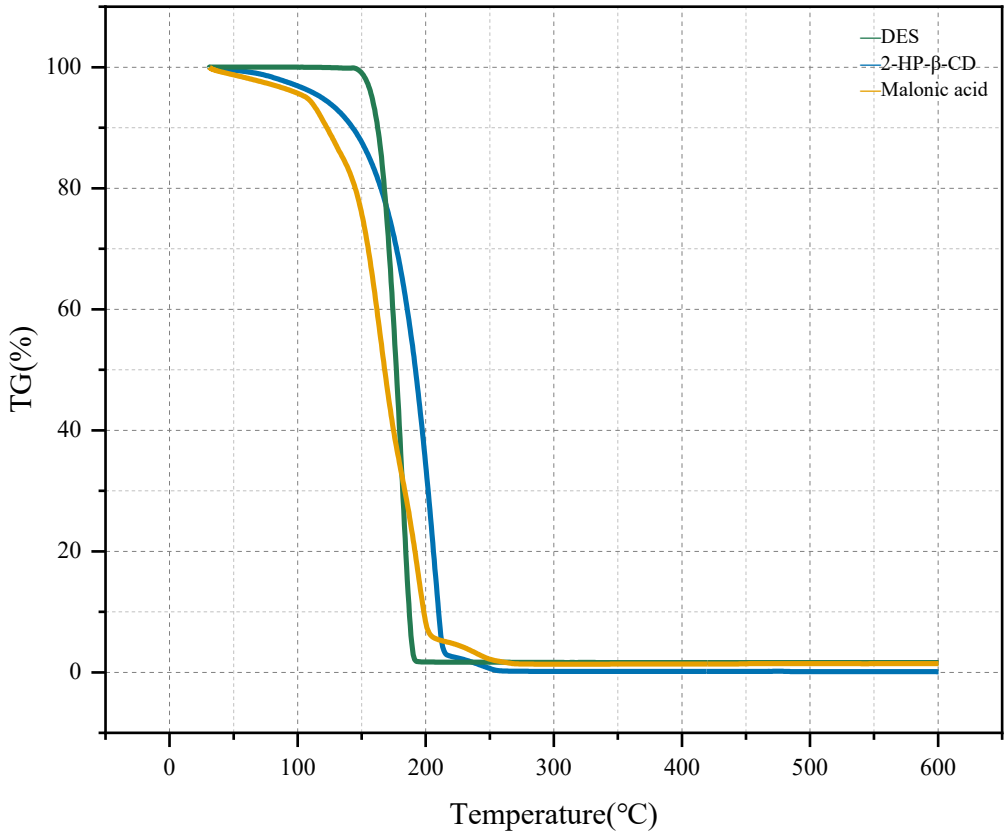


Fig. 3 TG curves of the DES and its individual components 2-HP-β-CD and Malonic acid.

Results and Discussion

Effect of DES type

To identify the optimal extraction conditions, four DESs with different compositions were prepared and evaluated. As shown in Fig. 4, DES-1 and DES-2 provided markedly higher extraction efficiency for GABA than DES-3 and DES-4, whereas DES-4 failed to achieve effective enrichment of GABA. A further comparison between DES-1 and DES-2 revealed consistently larger GABA peak areas for DES-2, indicating superior extraction performance and a more stable analytical response under identical operating conditions. Therefore, DES-2 was selected for subsequent experiments.

After selecting DES-2, its component mass ratio was further evaluated for GABA extraction. As depicted in Fig. 2(b), varying the component ratio of DES-2 led to an initial increase in GABA peak area followed by a plateau. The maximum peak area was obtained at a mass ratio of 1:2, which was significantly higher than those achieved under the other three ratios. This suggests that an appropriate increase in the donor to acceptor proportion is beneficial for enhancing solubilization and mass transfer within the DES microenvironment, thereby promoting the release and enrichment

of GABA[24]. By contrast, further increasing the ratio to 1:3 and 1:4 maintained relatively high peak areas but did not yield additional improvement compared with 1:2. This trend may reflect an approaching optimum in viscosity, polarity, and hydrogen-bonding network organization, beyond which additional gains become marginal. Considering extraction response and operational robustness, DES-2 at a mass ratio of 1:2 was selected for subsequent parameter optimization and method validation.

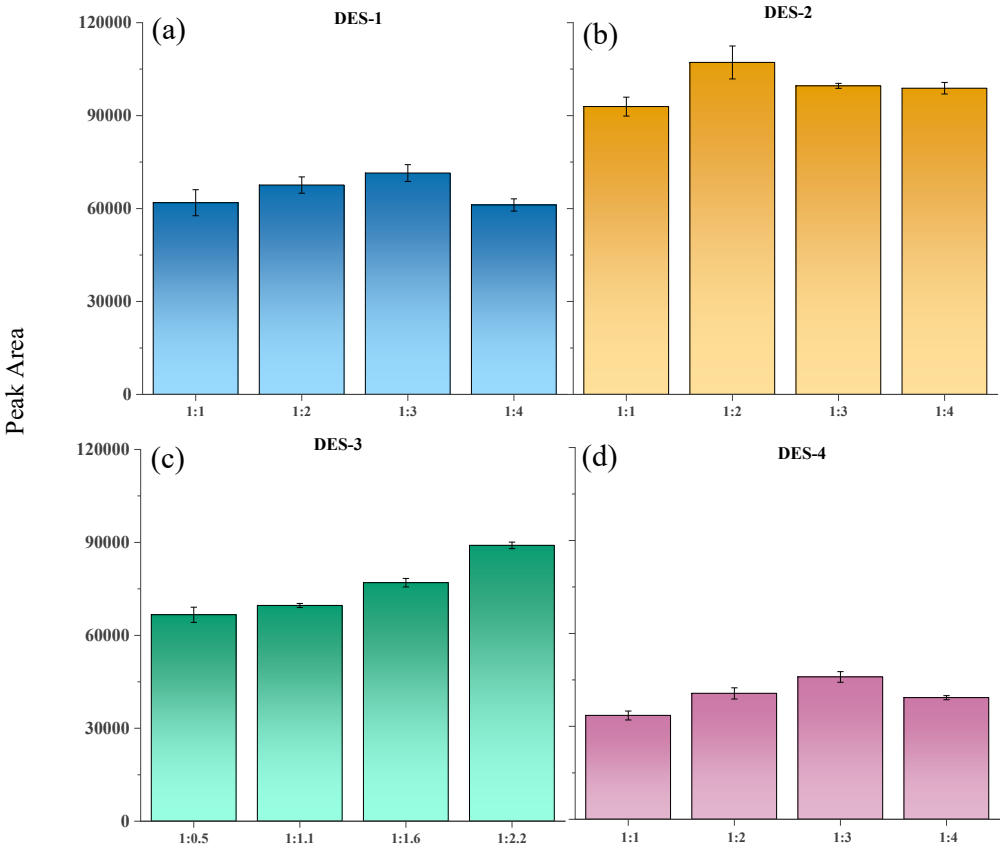


Fig. 4 Effect of different DES systems on the extraction performance of GABA.

Effect of the amount of DES

With a constant sample mass, an excessive volume of extractant may dilute the analyte in the extraction phase, whereas an insufficient amount may lead to incomplete extraction. To evaluate the influence of the amount of DES on extraction performance, 0.50, 0.75, 1.00, 1.25, and 1.50 mL of DES-2 were added to the samples following the proposed procedure. As shown in Fig. 5A, the peak area of GABA increased continuously as the amount of DES increased up to 1.00 mL. When the amount exceeded 1.00 mL, the peak area started to decrease. Therefore, 1.00 mL of DES-2 was selected for subsequent experiments to ensure a high peak area while minimizing extractant use.

Effect of ultrasonication time

Ultrasonication time strongly influences the mass transfer process of the target analyte from the matrix into the extraction phase[25]. Therefore, ultrasonication time was systematically investigated to achieve high extraction efficiency while keeping the pretreatment time as short as possible. With all other variables held constant, ultrasonication was performed for 10, 20, 30, 40, and 50 min. As shown in Fig. 5B, the peak area increased progressively as the ultrasonication time was extended from 10 to 40 min, indicating that appropriate sonication facilitates more complete release of the analyte and promotes its partitioning into the solvent phase. In contrast, the peak area started to decrease when the ultrasonication time was further prolonged to 50 min. This decrease may be associated with a temperature rise during extended sonication, which could induce partial hydrolysis or oxidation of the target compound. In addition, excessive sonication intensity or duration may compromise the stability of certain constituents, thereby lowering the effective recovery. Accordingly, 40 min was selected as the optimal ultrasonication time for subsequent experiments.

Effect of Solid/Liquid ratio

GABA extraction is strongly influenced by the solid-to-liquid ratio. Accordingly, solid-to-liquid ratios (0.2, 0.3, 0.4, 0.5, and 0.6 g/mL) were investigated. The volume of distilled water was fixed at 5 mL, and the ratio was adjusted by varying the mass of the tea sample. As shown in Fig. 5C, the solid to liquid ratio exerted a pronounced effect on GABA extraction. Increasing the ratio from 0.2 to 0.3 g/mL significantly reduced the peak area. The peak area then increased significantly at 0.4 g/mL, where the maximum value was obtained, but declined markedly as the ratio was further increased from 0.4 to 0.6 g/mL.This behavior can be attributed to the fixed extractant volume, under which excessive sample loading may cause insufficient wetting and an inadequate solid to liquid contact, thereby hindering complete transfer of the analyte and making it more difficult to reach extraction equilibrium. In addition, higher solid loading increases the apparent viscosity and reduces the fraction of free liquid phase, which can weaken ultrasonic cavitation and mass transfer intensification, ultimately compromising extraction performance[26].

Effect of K_2HPO_4 amount

Salt dosage had a significant effect on extraction performance. In this study, the effect of K_2HPO_4 on extraction efficiency was evaluated by testing 10, 11, 12, 13, and 14 g of K_2HPO_4, with emphasis on its interaction with the DES phase during salting out. As illustrated in Fig. 5D, the peak area of reached its maximum for GABA was achieved at 13 g of K_2HPO_4. At the K_2HPO_4 amount above 13 g, the excessively high ionic strength may increase the interfacial tension of the system, facilitating the accumulation of impurities at the phase boundary and thereby reducing the apparent yield of GABA[27]. Accordingly, 13 g of K2HPO4 was selected as the optimal salt amount for subsequent experiments.

Effect of derivatization time

To determine the optimal derivatization time, the influence of reaction duration (5, 10, 15, 20, and 25 min) on the peak area of GABA was systematically examined. As shown in Fig. 5E, the peak area reached its maximum at 20 min, and further extension of the reaction time resulted in negligible improvement. This indicates that the reaction between GABA and the derivatization reagent was nearly complete within 20 min, allowing the maximal formation of the derivative and thus the highest analytical response. Prolonged reaction time may also promote side reactions or lead to degradation of certain labile derivatives, which could undermine analytical accuracy[28]. This observation is consistent with previous reports indicating that the required derivatization time varies with reagent chemistry. For example, CNBF type reagents typically require tens of minutes, whereas more reactive reagents such as OPA generally proceed within a shorter time window. Therefore, 20 min was selected for subsequent experiments.

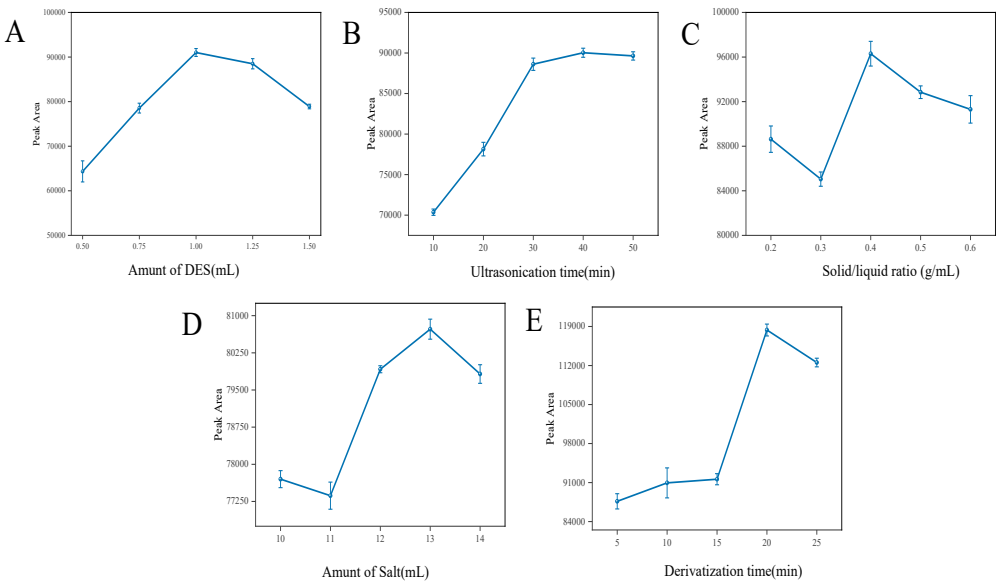


Fig. 5 Effects of key parameters on the extraction and determination of GABA:(A) Amount of DES; (B) Ultrasonication time; (C) Solid/liquid ratio;(D) Amount of K_2HPO_4; (E) Derivatization time.

Statistical analysis and model fitting

Based on the preliminary single-factor experiments, the extraction parameters were further optimized using a Box-Behnken design (BBD). The statistical significance and the interaction effects of the five independent variables are discussed below.

The relationship between the response and the independent variables is described by Eq. (6), where Y denotes recovery, and A, B, C, D, and E represent amount of DES ,ultrasonication time, solid to liquid ratio, amount of K_2HPO_4, and derivatization time, respectively.

(6) $Y = 91.00 + 1.67A + 2.00B + 1.82C + 1.93D + 1.61E + 0.3825AB - 0.3300AC + 0.0625AD +$

$$0.0200AE - 0.0100BC + 0.2600BD + 0.0300BE - 0.0975CD + 0.1550CE - 0.1875DE - 3.47A^2 - 3.18B^2 - 3.02C^2 - 3.07D^2 - 2.80E^2$$

As summarized in Table 2, the regression model was highly significant ($p < 0.0001$), indicating a robust association between the experimental factors and the response. Moreover, the lack of fit was not significant ($p = 0.9994 > 0.05$), suggesting that the model adequately describes the experimental data and can be used to predict the optimal conditions. The coefficient of determination (R2), adjusted coefficient of determination (Adjusted R^2), and the coefficient of variation (C.V.) were used to evaluate model fitness. The R^2 value of 0.9995 indicates that 99.95% of the total variability in recovery was explained by the model, while the Adjusted R^2 of 0.9991 further confirmed the excellent goodness of fit after accounting for the number of model terms. The low C.V. value further indicates satisfactory precision and reliability of the model fit.

Based on the p-values, all five linear terms (A–E) were extremely significant ($p < 0.0001$), indicating that the amount of DES, ultrasonication time, solid-to-liquid ratio, amount of K2HPO4, and derivatization time all significantly affected recovery. According to the F-values, the relative influence of these factors on recovery followed the order: ultrasonication time > amount of K2HPO4 > solid-to-liquid ratio > amount of DES > derivatization time.

Table 2 Analysis of the variance (ANOVA) for the second-order polynomial model.

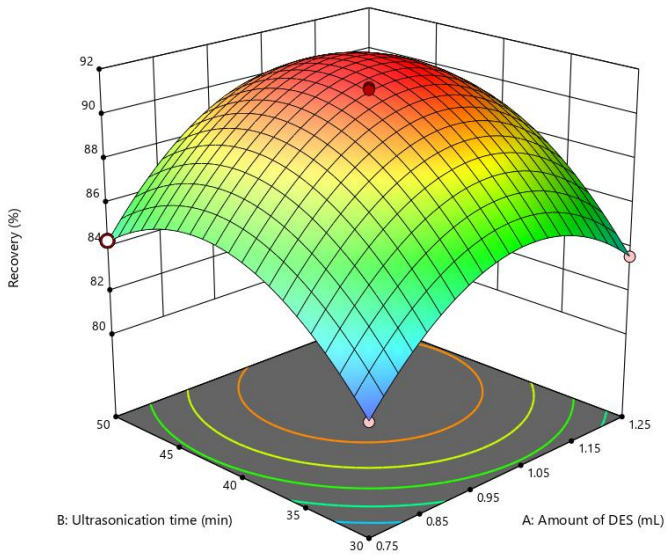
Source	Sum of squares	Degree of freedom	Mean square	F-value	P-value	Significance
Model	469.22	20	23.46	2478.12	< 0.0001	significant
A-Amount of DES	44.69	1	44.69	4720.36	< 0.0001	
B-Ultrasonication time	64.04	1	64.04	6764.31	< 0.0001	
C-Solid/liquid ratio	53.18	1	53.18	5617.27	< 0.0001	
D-Amount of Salt	59.87	1	59.87	6323.73	< 0.0001	
E-Derivatization time	41.25	1	41.25	4356.93	< 0.0001	
AB	0.5852	1	0.5852	61.82	< 0.0001	
AC	0.4356	1	0.4356	46.01	< 0.0001	
AD	0.0156	1	0.0156	1.65	0.2107	
AE	0.0016	1	0.0016	0.1690	0.6845	
BC	0.0004	1	0.0004	0.0423	0.8388	
BD	0.2704	1	0.2704	28.56	< 0.0001	
BE	0.0036	1	0.0036	0.3803	0.5430	
CD	0.0380	1	0.0380	4.02	0.0560	
CE	0.0961	1	0.0961	10.15	0.0038	
DE	0.1406	1	0.1406	14.85	0.0007	
A^2	105.31	1	105.31	11123.67	< 0.0001	
B^2	88.05	1	88.05	9299.94	< 0.0001	
C^2	79.66	1	79.66	8414.42	< 0.0001	
D^2	82.50	1	82.50	8714.12	< 0.0001	
E^2	68.32	1	68.32	7216.40	< 0.0001	

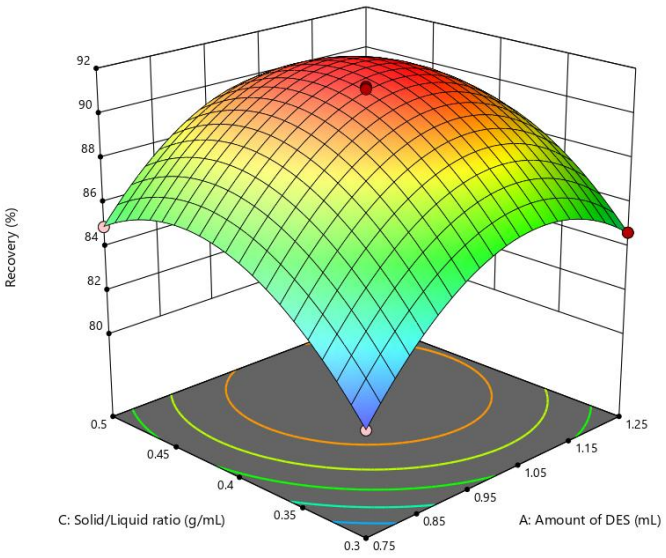
Residual	0.2367	25	0.0095			
Lack of Fit	0.0853	20	0.0043	0.1408	0.9994	not significant
Pure Error	0.1514	5	0.0303			
Cor Total	469.46	45				
Std. Dev.	0.0973	R^2	0.9995			
Mean	85.59	Adjusted R^2	0.9991			
C.V. %	0.1137	Predicted R^2	0.9988			
		Adeq Precision	156.9672			

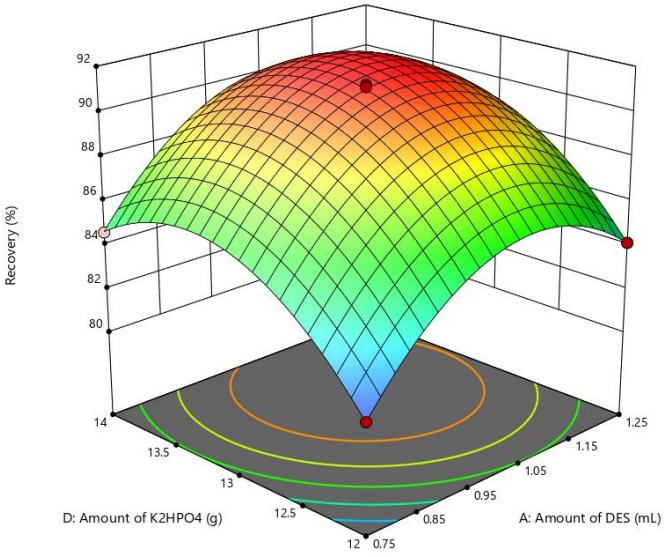
Interaction effects and determination of the optimal extraction conditions

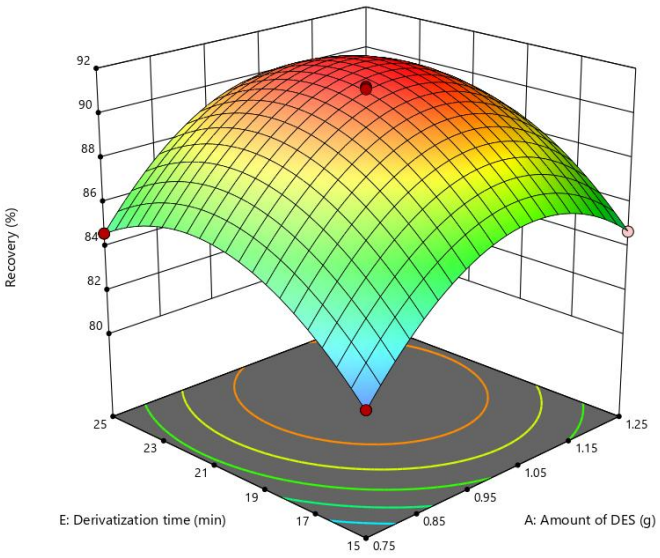
To further visualize the interactions among the factors and their effects on recovery, three-dimensional-response-surface plots were generated based on the fitted regression model, as shown in Fig. 6.

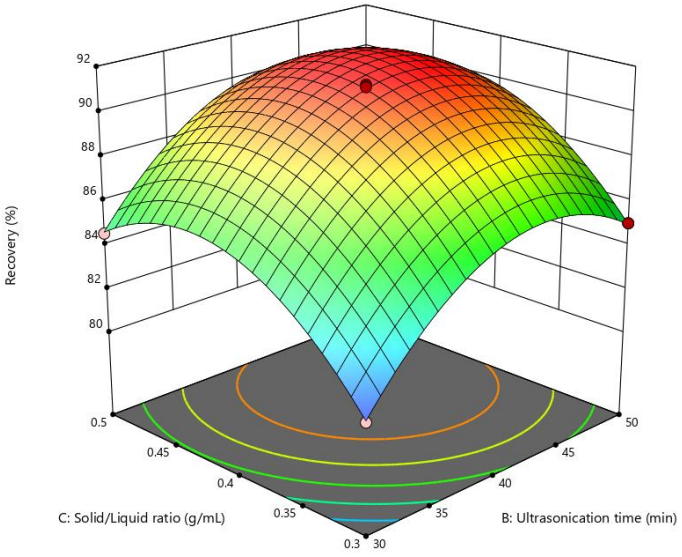
According to the Box-Behnken experimental results, the optimal conditions within the investigated ranges for maximizing GABA recovery were as follows: DES volume of 1.243 mL, ultrasonication time of 43.883 min, solid to liquid ratio of 0.425 g/mL, amount of K_2HPO_4 of 13.328 g, and derivatization time of 21.443 min. These conditions were in close agreement with those obtained from the one-factor experiments, further confirming the accuracy and reliability of the RSM-based model.

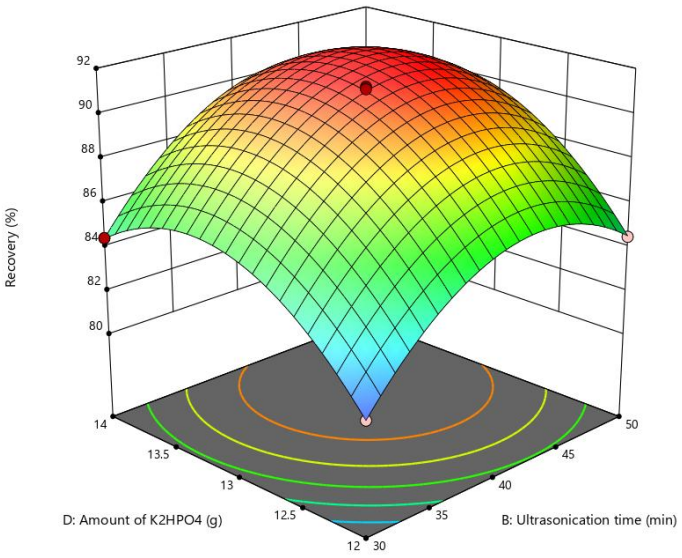


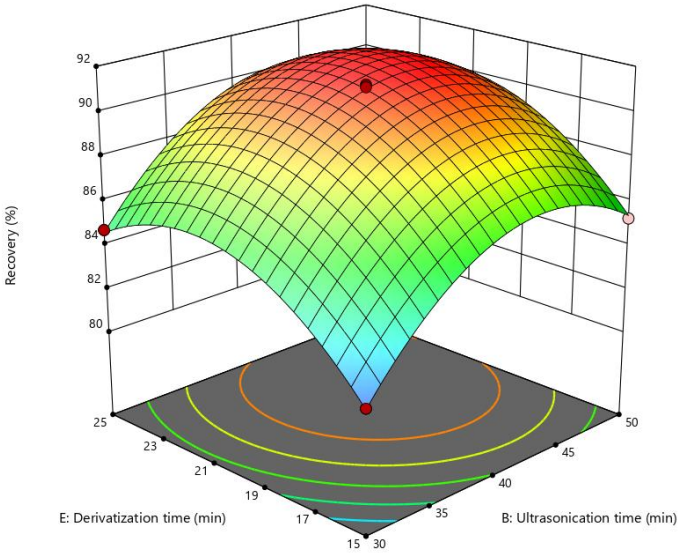

Recovery (%)
92
90
88
86
84
82
80
C: Solid/Liquid ratio (g/mL)
0.5
0.45
0.4
0.35
0.3
A: Amount of DES (mL)
0.75
0.85
0.95
1.05
1.15
1.25

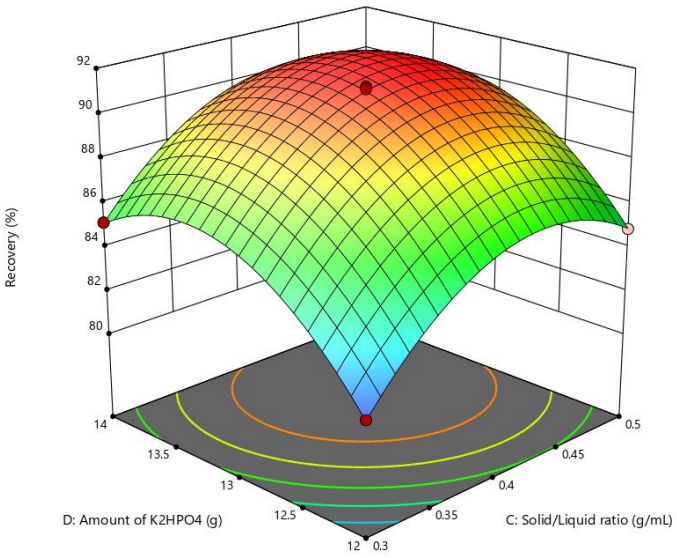

Recovery (%)
92
90
88
86
84
82
80
D: Amount of K2HPO4 (g)
14
13.5
13
12.5
12
A: Amount of DES (mL)
0.75
0.85
0.95
1.05
1.15
1.25

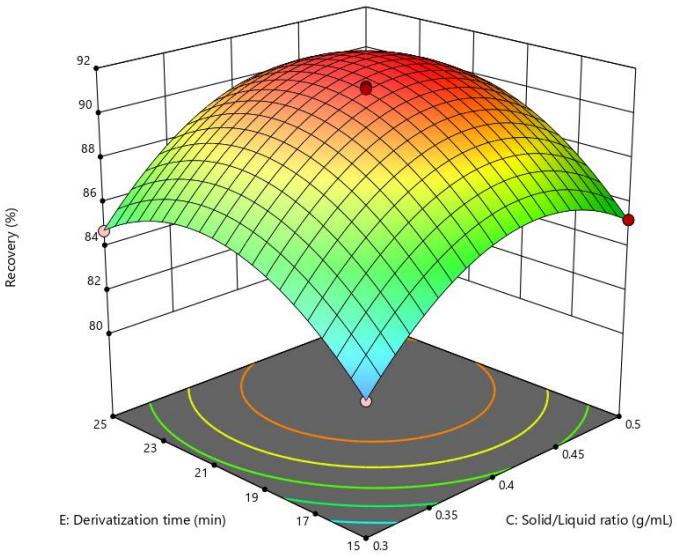

Recovery (%)
92
90
88
86
84
82
80
25
23
21
19
17
15
E: Derivatization time (min)
0.75
0.85
0.95
1.05
1.15
1.25
A: Amount of DES (g)

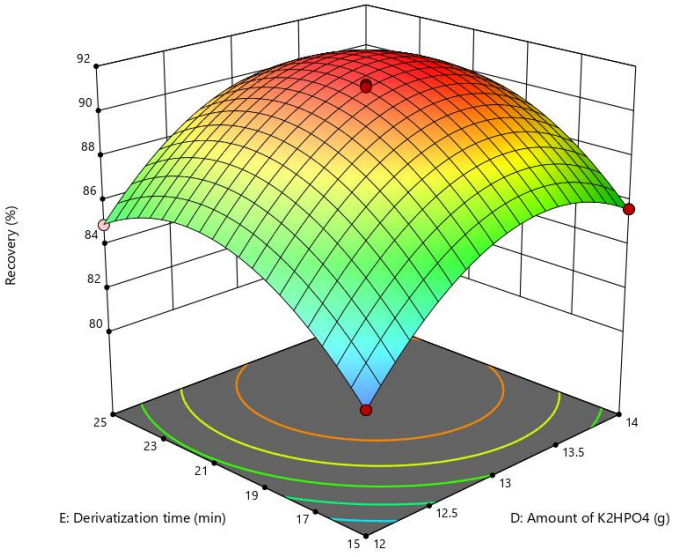

Recovery (%)
92
90
88
86
84
82
80
0.5
0.45
0.4
0.35
0.3
C: Solid/Liquid ratio (g/mL)
30
35
40
45
50
B: Ultrasonication time (min)


Recovery (%)
92
90
88
86
84
82
80
14
13.5
13
12.5
12
D: Amount of K2HPO4 (g)
30
35
40
45
50
B: Ultrasonication time (min)


Recovery (%)
92
90
88
86
84
82
80
25
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21
19
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15
E: Derivatization time (min)
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35
40
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50
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Recovery (%)
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13.5
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D: Amount of K2HPO4 (g)
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Recovery (%)
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E: Derivatization time (min)
0.3
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C: Solid/Liquid ratio (g/mL)


Recovery (%)
92
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88
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84
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80
25
23
21
19
17
15
E: Derivatization time (min)
12
12.5
13
13.5
14
D: Amount of K2HPO4 (g)

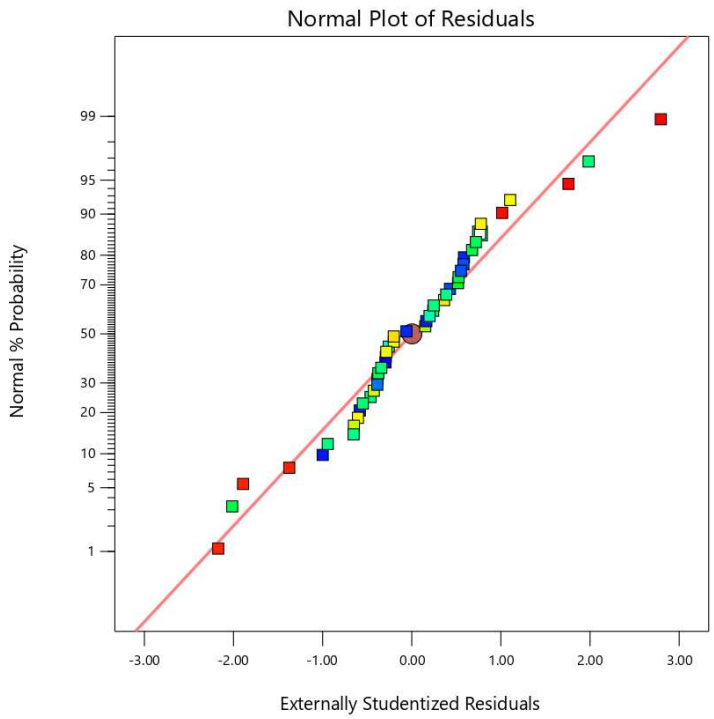


Fig. 6 Effects of five factors on the extraction recovery of GABA and comparison between the predicted and experimental values of the regression model.

Method validation

Linearity, LOD, LOQ and precision

To evaluate the applicability and analytical performance of the proposed method for GABA determination in tea, the key figures of merit were assessed under the optimized conditions. A series of GABA standard solutions (0.5, 2, 10, 50, 200 and 500μg/mL) was prepared and derivatized following the procedure described in the preceding section. An external calibration curve was constructed by peak area of the DABS-GABA derivative. The results are summarized in Table 3. Excellent linearity was obtained across the investigated range, with a coefficient of determination (R^2) of 0.9995. The limit of detection (LOD) and limit of quantification (LOQ) were 0.27 μg/mL and 1.25 μg/mL, respectively. In addition, the method exhibited satisfactory precision. Collectively, these validation results demonstrate that the SUPRADES-based approach enables robust and reproducible extraction and quantification of GABA in tea matrices.

Table 3 Figures of merit of the optimized method for the target analyte

Compound	LR(μg/mL)	Slopes	Intercepts	R^2	LOD (μg/mL)	LOQ (μg/mL)	Concentration (μg/mL)	RSD% (n =8)	RSD% (n =8)	Recovery %
								Intra-day	Inter-day	
GABA	0.5-500	18.82	127.08	0.9995	0.27	1.25	15	1.5	1.9	85.6%
							25	1.3	2.7	92.8%
							50	1.7	2.1	90.0%

Robustness

The robustness of the method was assessed by intentionally incorporating minor fluctuations in critical instrumental parameters: flow rate (0.7, 0.8, and 0.9 mL/min), column temperature (25, 28, and 33 °C), and injection volume (1.0, 1.5, and 2.5 μL). Compared with the optimal analytical conditions, the resulting variations in GABA quantification remained consistently below 2%. These findings indicate that the developed method possesses excellent robustness.

Real sample analysis

To demonstrate the practical applicability of the proposed method, the accuracy, repeatability, and contents of GABA in real tea samples were determined. The results are summarized in Table 4. Representative chromatograms of the tea samples and the standard solution are presented in Fig. 7.

Table 4 Results of GABA determination in tea samples

Samples	Content(μg.g^{-1})	Recovery (%)			ME %
		8μg/mL	30μg/mL	50μg/mL	
Taiwan GABA oolong tea	1565	89.5	95.3	93.4	90.4
Xinyang Maojian green tea	149	93.1	92.9	91	92.3
Jin Jun Mei black tea	123	95	102.3	96.8	95
Fuding white tea	168	96.3	97.5	104.3	96.1

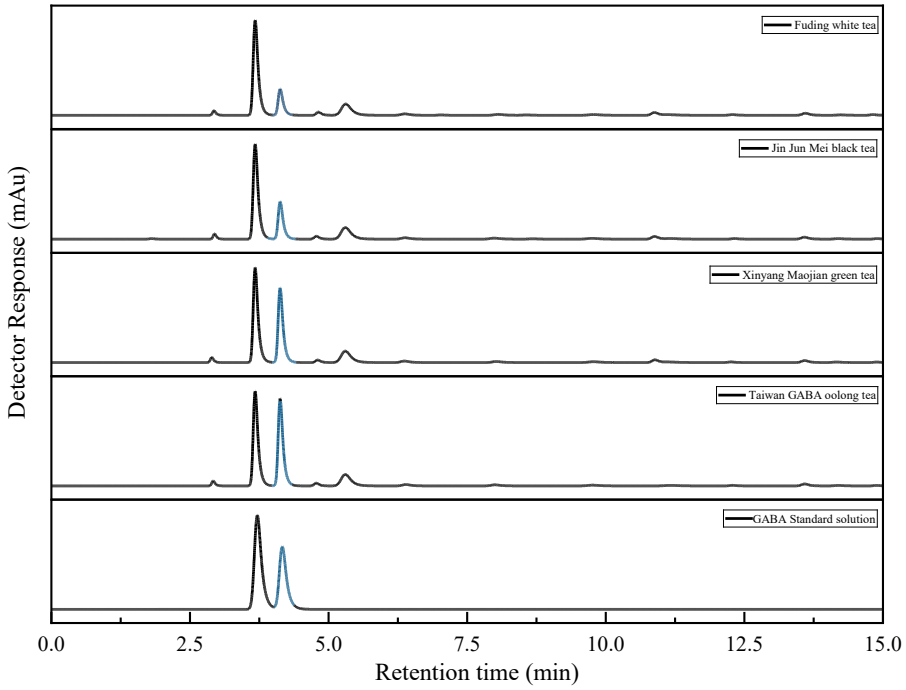


Fig. 7 HPLC chromatograms of standard solution and different tea samples. The peak highlighted in blue corresponds to GABA.

Note:The peak highlighted in blue corresponds to GABA.

Comparison with other methods

Table 5 compares the SUPRADES-UAE method coupled with salting-out assisted separation with previously reported procedures for determining GABA in tea and other food matrices.For tea, Horanni et al. [29] used aqueous brew extraction followed by FMOC-Cl derivatization and HPLC–UV detection, achieving LODs of 0.057–0.534 μg/mL and LOQs of 0.235–1.849 μg/mL, together with recoveries above 91%. Unlike conventional aqueous extraction, our approach employs a supramolecular DES combined with ultrasound assistance and salting out separation, allowing extraction and phase separation to be integrated into a single workflow for tea samples.Under the optimized conditions, the method provided a linear range of 0.5–500 μg/mL, with an LOD of 0.27 μg/mL and an LOQ of 1.25 μg/mL.

Across various food matrices, Pencheva et al. [30] combined simple extraction and filtration with dansyl chloride derivatization and UHPLC–UV, reporting a linear range of 2–1000 μg/mL, a limit of detection of 0.35 μg/mL, a limit of quantification of 1.15 μg/mL, and relative standard deviations of 0.73–4.64 %. Zhuang et al. [31] developed an FMOC-Cl–HPLC–UV method for fermented soy products, with a linear range of 1–100 μg/mL, a limit of detection of 0.30 μg/mL, a limit of quantification of 1 μg/mL, and relative standard deviations below 5 %. Compared with these LC-based methods, our approach provides comparable sensitivity, with a limit of detection of 0.27

μg/mL, while maintaining good repeatability, with relative standard deviations no higher than 2.7 %. These results highlight the stabilizing contribution of the supramolecular solvent microenvironment together with salting-out assisted separation. In addition, Xiao et al. [32]used DABS-Cl derivatization with HPLC–DAD for candy beverage products, reporting a linear range of 1–100 μg/mL, an R^2 value of 0.9995, and relative standard deviations of 0.99–3.93 %. In the present work, limits of detection and quantification were also established for the tea matrix, and recoveries of 85.6–92.8 % were achieved with relative standard deviations no higher than 2.7 %.

Table 5 Comparison between this method and other extraction methods（n=5）

Reference	Matrix	Sample preparation	Derivatization	Separation	LR (μg/mL)	LOD(μg/mL)	LOQ (μg/mL)	Recovery / Precision
[29]	Tea	Aqueous brew extraction	FMOC-Cl	HPLC-UV	NR	0.057–0.534	0.235–1.849	Recovery > 91 %
[30]	Plant foods and medicinal plants	Filtration	Dansyl chloride	UHPLC-UV	2-1000	0.35	1.15	GABA recovery 102.6–104.9 %; RSD 0.73–4.64%
[31]	Fermented soy products	80% EtOH UAE with centrifugation	FMOC-Cl	HPLC-UV	1-100	0.3	1	Recovery 83.8–100.8%; RSD < 5%
[32]	Candy beverage products	Water UAE with centrifugation	DABS-Cl	HPLC-DAD	1–100	NR	NR	Recovery 93.95–103.90%; RSD 0.99–3.93%
This work	Tea	Supramolecular DES based UAE; salting out separation	DABS-Cl	HPLC-UV	0.5-500	0.27	1.25	Recovery 85.6–92.8%; RSD ≤ 2.7%

Greenness assessment of the method

The environmental sustainability of the developed protocol was comprehensively evaluated using two complementary quantitative metrics: the Analytical Eco-Scale (AES) and the AGREE (Analytical GREENness) index.

As summarized in Table 6, the AES provides a quantifiable measure of the method's greenness by auditing parameters such as the type and quantity of reagents, energy consumption of instrumentation, occupational hazards, and waste generation. This scoring system operates on a penalty point (PP) basis, where deductions are subtracted from an ideal score of 100. According to our assessment, the final Eco-Scale score for the proposed method was 75[33]. The primary penalties were attributed to the requisite use of organic modifiers (specifically acetonitrile and methanol) and the inevitable generation of analytical waste. According to the AES classification criteria, a score of 75 or higher is considered to indicate an excellent green analytical method.Consequently, the current method exhibited favorable greenness according to the AES evaluation, a result largely facilitated by the implementation of non-toxic DES as sustainable alternatives to conventional extraction media, coupled with the rigorous optimization of reagent

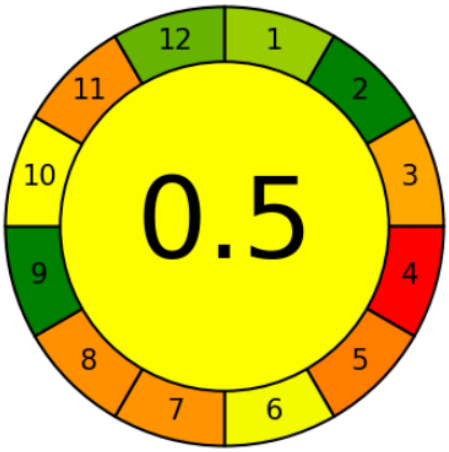
consumption.

To further validate the sustainability of the developed method, the AGREE metric based on the twelve principles of Green Analytical Chemistry (GAC)—was applied. Pena-Pereira and co-workers[34] further noted that, for a comprehensive sustainability assessment with an emphasis on sample preparation, the AGREE framework can be constructed around ten impact categories and subsequently recalculated into sub-scores on a 0–1 scale; the results are visualized as a pictogram in Fig. 8(a). A red–yellow–green color gradient reflects the method's performance for each principle, while the width of each sector represents the weight assigned to that criterion. The covered categories include: (1) sample treatment, (2) sample amount, (3) device positioning/in situ measurement, (4) sample preparation stages, (5) automation and miniaturization, (6) derivatization, (7) waste, (8) analytical throughput, (9) energy consumption, (10) reagent sources, (11) toxicity, and (12) operator safety. As shown in Fig. 8(b), the detailed scoring criteria are provided in a table. The AGREE evaluation results of this study, as shown in Fig. 8, indicated that the proposed method achieved an overall score of 0.49. Although the overall score was 0.49, indicating moderate greenness, the method still showed limitations in several criteria under the AGREE framework. Consequently, under the more stringent AGREE framework, the method could not be classified as fully green under the AGREE framework, reflecting a persistent ecological footprint. The observed discrepancy between the results stems from the fundamental differences in evaluation criteria: while the AES emphasizes linear deductions based on chemical consumption and inherent hazards,the AGREE framework imposes more holistic requirements regarding automation, in situ measurement, system integration, and occupational safety. Drawing from this assessment, the proposed method offers several avenues for future refinement and optimization:

To enhance the method's performance within the AES framework, priority should be given to mitigating waste generation and reducing the energy intensity of the ultrasonic water bath. Regarding the AGREE metric, further refinements should focus on streamlining sample pretreatment stages, minimizing organic solvent consumption, and simplifying or eliminating derivatization steps.

In summary, while the AES affirms the greenness of the proposed protocol in terms of fundamental reagent consumption, the AGREE assessment reveals inherent limitations concerning systemic sustainability, health, and occupational safety.

(a)



1. Sample treatment

2. Sample amount

3. Device positioning

4. Sample preparation stages

5. Automation and miniaturization

6. Derivatization

7. Waste

8. Analysis throughput

9. Energy consumption

10. Source of reagents

11. Toxicity

12. Operator's safety

(b)

Criteria	Score	Weight
1.Direct analytical techniques should be applied to avoid sample treatment.	0.7	2
2.Minimal sample size and minimal number of samples are goals.	1.0	2
3.If possible, measurements should be performed in situ.	0.33	2
4.Integration of analytical processes and operations saves energy and reduces the use of reagents.	0.0	2
5.Automated and miniaturized methods should be selected.	0.25	2
6.Derivatization should be avoided.	0.52	2
7.Generation of a large volume of analytical waste should be avoided, and proper management of analytical waste should be provided.	0.29	2
8.Multi-analyte or multi-parameter methods are preferred versus methods using one analyte at a time.	0.29	2
9.The use of energy should be minimized.	1.0	2
10.Reagents obtained from renewable sources should be preferred.	0.5	2
11.Toxic reagents should be eliminated or replaced.	0.28	2
12.Operator's safety should be increased.	0.8	2

Fig. 8 Greenness assessment of the analytical method based on the AGREE tool

(a) AGREE pictogram (b) Detailed evaluation criteria table

Table 6 Detailed Scoring of the Analytical Eco-Scale

Penalty points (PPs)					
Reagent	Amount PP	Hazard PP	Total PPs (Amount PP×Hazard PP)	Instrument	PPs
DES (2-HP-β-CD/malonic acid) (<10 mL)	1	0	0	Centrifuge	1
K_2HPO_4 (13 g)	2	0	0	Ultrasonic bath	2
Methanol (SPE conditioning) (<10 mL)	1	6	6	Vortex	1
DABS-Cl derivatization reagent (0.4 mL)	1	2	2	Water bath (70 °C)	2
Acetonitrile (mobile phase) (<10 mL)	1	4	4	HPLC	2
NaHCO3 solution (0.2 mL)	1	0	0	Occupational hazard	0
				Waste	5
Subtotal PP	-	-	12		13
Total PPs	-	-	25		
Analytical Eco-Scale score			75		

Note: Amount PP was assigned based on the amount used per sample/run; Hazard PP was assigned according to GHS classification in SDS. Total PPs = Amount PP × Hazard PP. Analytical Eco-Scale score = 100 − Total PPs.

Conclusions

In this study, a SUPRADES prepared from 2-HP-β-CD and malonic acid was combined with ultrasound-assisted extraction and salting-out-assisted phase separation to enable efficient enrichment of GABA prior to HPLC analysis. The proposed workflow showed good model predictability and satisfactory analytical performance, including high linearity, low LOD/LOQ, acceptable intra-/inter-day precision, and reliable recoveries in real tea samples.Moreover, the method exhibited only a limited matrix effect, supporting its practical applicability for routine quantification of GABA in complex tea matrices. Greenness assessment suggested that the protocol performs well in terms of fundamental reagent consumption (Eco-Scale), while further improvements are still possible under the more stringent AGREE framework, particularly by simplifying pretreatment steps and reducing organic solvent and derivatization dependence. Overall, this SUPRADES based strategy provides a practical and tunable sample preparation route for polar bioactives in tea and can be extended to other complex food matrices by tailoring the HBA and HBD components.

Declarations

Ethical approval This article does not contain any studies with human participants or

animals performed by any of the authors.

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Conflict of interest The authors declare no competing interests.

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