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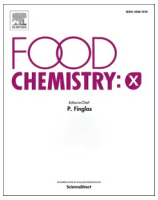

A green deep eutectic solvent-based aqueous two-phase system combined with chemometrics for flavonoids extracting and detecting in honey

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ABSTRACT

In this study, an eco-friendly and designable aqueous two-phase system (ATPS) was developed using natural deep eutectic solvents (DES) and short-chain alcohols. The formation mechanism and influence of various factors on phase behavior were investigated. Optimal extraction parameters were determined through single-factor experiments and response surface methodology (RSM): 45 °C temperature, 45 % *n*-propanol, 40 % DES, and 0.06 mL quercetin working standard solution. Based on the alternating trilinear decomposition assisted multiple curve resolution (ATLD-MCR) algorithm, a calibration model was established for simultaneous and rapid quantitative analysis of flavonoids in Acacia honey, achieving 74.0–86.6 % accuracy and 0.82–2.20 % standard deviation. Moreover, the green chemistry metrics of development method was evaluated using analytical greenness (AGREE) and compared with earlier published methods in the literature. The results indicated that this novel combination strategy of DES-based ATPS with chemometrics conforms to the principles of green chemistry, and is suitable for extracting active components from plants.

1. Introduction

Honey is a natural sweetener that is produced, sold, and consumed globally. It possesses antioxidant, anti-inflammatory, and antibacterial properties, giving it high nutritional value and health benefits, and is therefore used for therapeutic purposes(Ilia, Simulescu, Merghes, & Varan, 2021; Seraglio et al., 2021; Zammit Young & Blundell, 2023). The biological activities of honey mainly depend on the composition and content of compounds it contains, including flavonoids, phenolic acids, enzymes, pigment, and alkaloid(Valverde, Ares, Stephen Elmore, & Bernal, 2022). Among these, flavonoids play a significant role, accounting for more than 50 % of all natural phenolic compounds in honey (Silva et al., 2021; Zhang, Wang, Liu, Qing, & Nie, 2024). However, due to the wide variety and relatively low content of flavonoids, achieving efficient extraction and accurate analysis presents various challenges. Numerous studies in the literature have addressed these challenges. In an earlier study, liquid-liquid extraction (LLE) and HPLC were applied to analyze phenolic acids and flavonoids(Kıvrak & Kıvrak, 2016). Another study employed sugaring-out assisted liquid-liquid extraction (SULLE) combined with HPLC-electrochemical detection to extract and analyze phenolic compounds in honey(Zhu et al., 2019). Furthermore, Silva et al., conducted a study using a QuEChERS technique followed by HPLC/DAD to quantify phenolic compounds from Mimosa scabrella Bentham honeydew honey(Silva, Gonzaga, Fett, & Oliveira Costa, 2019). However, traditional extraction techniques combined with chromatography-based methods present certain challenges when using real honey samples. These challenges include high costs, time consumption, the use of large amounts of harmful solvents, complex chromatographic conditions, and inaccurate quantitative results, all of which do not conform to the principles of Green Analytical Chemistry (Rutkowska, Płotka-Wasylka, Sajid, & Andruch, 2019; Vian, Breil, Vernes, Chaabani, & Chemat, 2017; Zhang et al., 2024).

Deep eutectic solvents (DESs) are a type of green solvents with significant research relevance. They offer various advantages such as low cost, easy preparation, and relatively wide liquid range(Alshammari, Almulgabsagher, Ryder, & Abbott, 2021; Oke & Ijardar, 2021; Qin et al., 2022). Additionally, they exhibit green characteristics including chemical stability, low vapor pressure, low toxicity, excellent

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biocompatibility, and structural designability(Farias, Pereira, Coutinho, Igarashi-Mafra, & Mafra, 2020; Marchel, Coroadinha, & Marrucho, 2020). These properties make DESs as a new generation of "green solvents" that can replace conventional solvents and ionic liquids (ILs), which are widely used in synthesis, catalysis, analytical chemistry, and biomedicine(Hong et al., 2020; Ji & Lv, 2020; van Osch et al., 2017). The characteristics of DESs, such as extensive hydrogen bonding, robust electrostatic interactions, and significant partial van der Waals forces, have propelled their popularity as efficient natural product extractants (Van Osch, Dietz, Warrag, & Kroon, 2020; Wang, Liu, Zhao, Wang, & Yu, 2019), have attracted significant interest in applications for green extraction and enrichment of bioactive components from plant-based products(He et al., 2024; Rashid, Mohd Wani, Manzoor, Masoodi, & Masarat Dar, 2023; Sportiello et al., 2023).

The aqueous two-phase system (ATPS) extraction is a more recent liquid-liquid extraction (LLE) technology that has been successfully used to extract active compounds from plants(Cai, Ma, Li, & Tan, 2024; Mashhaditafreshi & Haghtalab, 2024). High extraction yields and product purity can be achieved using ATPS, while maintaining the biological activity of the extracted compounds(Enriquez-Ochoa, Sánchez-Trasvia, Hernández-Sedas, Mayolo-Deloisa, & Valdez-Garcia, 2020; Mashhaditafreshi & Haghtalab, 2024). In contrast to the conventional LLE technique, the ATPS extraction excels with its gentle extraction conditions, minimal sample volume demands, a spectrum of intrinsic regulatory factors, rapid phase separation, straightforward operation and scalability, superior extraction efficiency, and its adaptability for large-scale industrial production(Enriquez-Ochoa et al., 2020; Zhang et al., 2024).

Efficient extraction methods, when paired with suitable instrumental techniques, are pivotal in the comprehensive examination of flavonoids present in honey. High performance liquid chromatography coupled with diode array detection (HPLC-DAD) emerges as a formidable analytical instrument, renowned for its high resolution and the wealth of data it provides(Zhang et al., 2021). However, the data array obtained from HPLC-DAD contains not only valuable chemical information, but also responses to unknown interference, instrument noise, and background. Therefore, the choice of chemometric algorithms to process and analyze these complex high-dimensional data is crucial to the success of chromatography-based methods. The alternating trilinear decomposition-assisted multivariate curve resolution (ATLD-MCR) algorithm is a recently developed multivariate calibration algorithm that utilizes the combined advantages of ATLD and MCR algorithms, and also possesses the "second-order advantage"(Wang et al., 2019). It can accurately extract qualitative and quantitative information from analytes with peak overlap, unknown interference, and retention time drift. It has been widely used for the qualitative and quantitative analysis of multi-components complex systems using chromatography. In this study, an analytical method was developed for the simultaneous extraction and detection of flavonoids in honey using DES-based ATPS and chemometric-enhanced chromatography. As shown in Fig. 1, the accuracy and greenness of the method were enhanced using three key phases: natural DESs, ATPS extraction, and chemometric data processing. Three natural DESs were synthesized using betaine as a hydrogen bond acceptor and D-(+)-glucose, D-sorbitol, and xylitol as hydrogen bond donors. Additionally, designable ATPSs were developed using DESs and short-chain alcohols. The chemometric-enhanced HPLC-DAD method was then used for the simultaneous extraction and quantitation of flavonoids in Acacia honey. Finally, using the AGREE calculator, the green chemistry metrics of development method were evaluated and compared with the published methods in the literature. The analytical methodology employed in this study adheres closely to the tenets of green chemistry, promising broader applications for the efficient extraction and precise qualitative and quantitative analysis of bioactive compounds within plant-based products.

2. Materials and methods

2.1. Honey samples and chemical reagents

The HPLC-grade standards, including myricetin, fisetin, quercetin, hesperidin, and kaempferol, each with a purity of no less than 98 %, were sourced from Aladdin Biochemical Technology Co., Ltd., a reputable supplier based in Shanghai, China. The hydrogen-bond acceptor (betaine) and hydrogen-bond donor (D-(+)-glucose, D-sorbitol, and xylitol), each with a purity of not less than 98 % were procured from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Methanol (HPLC grade) was procured from Thermo Fisher Scientific Inc. (USA). Analytical-grade ethanol, *n*-propanol (NPA), *iso*propanol (IPA), and formic acid were obtained from Aladdin (Shanghai, China) and Sinopharm Chemical Reagents (Beijing, China). Acacia honey was obtained from Henan Zhuoyu Bees Industry Co. Ltd. (Changge, China). In this study, deionized water was obtained using Milli-Q integral water

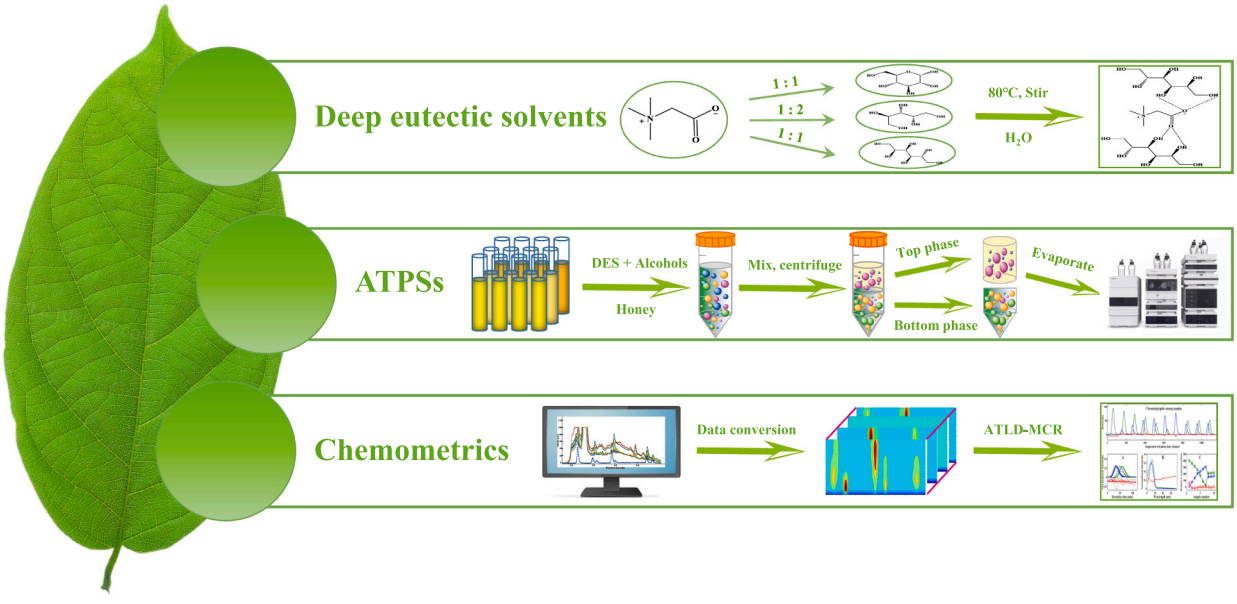

Fig. 1. Shematic diagram of the DES-based ATPS combined with chemometrics for simultaneous extraction and determination of flavonoids in honey.

purification system (Millipore Company, USA).

2.2. Sampling and preparation

The stock solutions were prepared by dissolving the standards in methanol and storing them in amber-colored bottles at 4 °C. The respective concentrations were as follows: myricetin at 1990 μg/mL, fisetin at 2060 μg/mL, quercetin at 2040 μg/mL, hesperidin at 2030 μg/mL, and kaempferol at 2030 μg/mL. Working standard solutions were freshly prepared by diluting the stock solutions in the mobile phase as required. The mobile phase consisted of 0.1 % formic acid in methanol and 0.1 % formic acid in water in a ratio of 75: 25 (*v*/v).

A calibration set of six varying standard concentrations was prepared by adding a specified volume of the working standard solution to a 10 mL amber-colored volumetric flask and made up to volumes using the mobile phase. The concentration of each analyte was randomly designed within its linear range, and a maximum was intended to produce a chromatographic response of approximately 20 mAu. In addition, as shown in Table S1S cross-design was performed for analytes with similar structures to avoid interference.

For ATPS experiments, the standard solution mixture was prepared by adding appropriate volumes of standard stock solutions of five flavonoids to a 10 mL volumetric flask, and diluted to volumes using methanol. The concentrations of the various standards prepared were as follows: myricetin at 3.18 × 10^2 μg/mL, fisetin at 6.18 × 10^2 μg/mL, quercetin at 3.26 × 10^2 μg/mL, hesperidin at 2.03 × 10^2 μg/mL, and kaempferol at 2.03 × 10^2 μg/mL. The sample stock solution was prepared by adding 15 g of Acacia honey to a 25 mL ambered-color volumetric flask, and diluted to volumes using mobile phase. A honey pretreatment solution was obtained by sonicating the solution at 45 °C for 20 min using an ultra sonicator, and then placing it in a refrigerator at 4 °C for subsequent ATPS experiments.

2.3. Synthesis and characterization of deep eutectic solvents

The preparation methods of DESs are usually simple and do not generate by-products, including direct heating method, freeze-drying method, grinding method, microwave-assisted method, ultrasound-assisted method, etc.(Oke & Ijardar, 2021; Van Osch et al., 2020). In this work, betaine was chosen as the hydrogen-bond acceptor, and D-(+)-glucose, D-sorbitol, and xylitol were used as hydrogen-bond donors. Three DESs were synthesized according to the proportions shown in Table S2. After simple mixing, stirred in an 80 °C water bath for 1 h until a uniform and transparent liquid was formed.

The FT-IR and ^{1}H NMR spectra of three DESs are shown in Fig. S1 and S2. It can be seen that the absorption bands of the -OH groups in the three DESs correspond to broad peaks around 3400 cm^{-1} and 1600 cm^{-1}, respectively, which further confirms the existence of hydrogen bonds. Moreover, the functional groups of the reactants remained stable during the synthesis process, and no new peaks appeared, indicating that no chemical reaction occurred in the preparation of DESs. In summary, the formation of the three DESs is mainly due to the hydrogen bonding between HBA and HBD.

2.4. Aqueous two-phase extraction

2.4.1. Phase diagrams determination

In this study, an aqueous two-phase system was constructed using three synthesized DESs with *n*-propanol (NPA) and *iso*propanol (IPA). The phase diagram of the DES-alcohol was prepared using turbidity titration at room temperature and atmospheric pressure. Initially, a DES aqueous solution with a mass fraction of 80 % was prepared to reduce viscosity and facilitate titration. Subsequently, a certain amount of DES was dropped into a test tube, weighed, and the total mass of the test tube and aqueous solution was recorded. Further, the alcohol was added dropwise, and mixed thoroughly using a vortex oscillator until turbidity was observed. The weight of each component in the mixture was measured and calculated. The mixed system was made clear by adding water, and the weight of each component in the system was recorded. These steps were repeated until the formation of turbidity was no longer possible. Using the data, the bimodal curves were obtained and fitted in Merchuk's equation(Moradi, Mohammadian, Pazuki, & Shahriari, 2022), where the *x*-axis and *y*-axis represented the mass fractions of alcohols and DESs in the mixed system, respectively.

$$[\mathrm{DES}] = \mathrm{A} \times exp\left[\left(\mathrm{B} \times [\mathrm{Alcohol}]^{0.5}\right) - \left(\mathrm{C} \times [\mathrm{Alcohol}]^{3}\right)\right] \quad (1)$$

where [DES] and [Alcohol] are the weight percent of three DES (i.e. Bet-Glu, Bet-Sor, Bet-Xyl) and two short-chain alcohols (i.e. NPA and IPA) in the systems, respectively.

2.4.2. Experimental design and response surface methodology optimization

As shown in Table S3, a certain amount of DESs, alcohols, and quercetin standard solutions were mixed to form ATPSs, and the optimal phase-formation of DESs and alcohols was determined based on the extraction rate of quercetin. Employing a single-factor experimental design, this study meticulously examined the influence of various experimental parameters on the partitioning behavior of flavonoids for ATPSs. Referring to the literatures(Cai et al., 2019; Enriquez-Ochoa et al., 2020), the factors for investigation are including DES concentration (40 %, 46 %, 52 %, 58 %, 64 %), NPA concentration (24 %, 30 %, 36 %, 42 %, 48 %), temperature (25 °C, 35 °C, 45 °C, 55 °C, 65 °C) and additional amount of quercetin (6.53 μg, 9.79 μg, 13.06 μg, 16.32 μg, 19.58 μg), respectively. The specific operation method of single factor experiments was described in detail in the **Supporting Information**. The optimal extraction parameters were ascertained through the application of the Box-Behnken Design (BBD) of Response Surface Methodology (RSM), utilizing Design Expert software, Version 12, developed by Stat-Ease Inc., Minneapolis, MN. Based on the variance of single factor experiments, a response surface experiment was designed at a fixed temperature of 45 °C, with an additional amount of quercetin (A), NPA concentration (B), and DES concentration (C) as independent variables, and the extraction yields of quercetin (*Y*) as the response factor. As delineated in Table 4S, the experimental design encompassed 5 central points per block, encompassing 3 quantitative factors, and was structured to execute a total of 17 experimental runs. The optimal parameters were ascertained by employing the mathematical model presented in Eq. (2).

$$Y = B_0 + \sum_{i=1}^{3} B_i X_i + \sum_{i=1}^{3} B_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{3} B_{ij} X_i X_j + \sum_{i=1}^{3} \sum_{j=i+1}^{3} B_{iij} X_i^2 X_j \quad (2)$$

where Y represents the response variable; X_i and X_j denote the independent variables under investigation. The constants B_0, B_i and B_{ii} correspond to the intercept, linear coefficients, and quadratic coefficients, respectively. Additionally, B_{ij} and B_{iij} represent the interaction coefficients for the cross-product terms.

2.4.3. Partitioning of flavonoids in ATPS

The two-phase extraction experiment involved the following stages. Firstly, a certain volume of quercetin standard solutions or honey pretreatment solution, DESs, and alcohols were added to a 10 mL centrifuge tube, and vortexed thoroughly. Secondly, the mixture was sonicated continuously at 45 °Cfor 20 min with an ultrasonic power of 220 W, and allowed to settle for 30 min. Thirdly, the solution was centrifuged for 10 min at 45 °C and 10,000 rpm, resulting in separation into top and bottom phases, and the volume of each phase was recorded. Furthermore, 1 mL of the top phase was carefully transferred using a micropipette, concentrated using nitrogen as the blowing agent and diluted to 1 mL with the mobile phase. Finally, the solutions were filtered through 0.22 μm filter membranes and analyzed using an HPLC-DAD system. The optimal ATPS was selected based on the extraction yield of quercetin in

the top phase. Each sample was independently analyzed in triplicate, with the mean value being utilized to assess the influence of multiple factors on the partitioning behavior of flavonoids of the ATPSs. The phase volume ratio (V_R) of ATPSs and the extraction yield (Y, %) of quercetin were quantitatively determined through the application of the subsequent equations:

$$V_R = \frac{V_{top}}{V_{bottom}} \tag{3}$$

$$Y\% = \frac{[A]_{top} V_{top}}{[A]_{top} V_{top} + [A]_{bottom} V_{bottom}} \times 100 \tag{4}$$

where $[A]_{top}$ and $[A]_{bottom}$ (μg/mL) are the contents of quercetin in the top and bottom phases, and V_{top} and V_{bottom} (mL) are the volumes of ATPS in the top and bottom phases, respectively.

2.5. Chromatographic methods

The chromatographic analysis was conducted utilizing a 1260 liquid chromatography system from Agilent Technologies, USA. The separation was achieved on an Agilent Eclipse XDB-C18 column (4.6 mm × 250 mm, 5.0 μm) under ambient temperature conditions, with the mobile phase delivered at a flow rate of 1 mL/min. The isocratic elution was used, and the mobile phase consisted of methanol and water in a ratio of 75: 25 (*v*/v). Referring to literatures(Alfaris, Wabaidur, Alothman, Altamimi, & Aldayel, 2020; Zhang et al., 2021), 0.1 % formic acid was added in the mobile phase. The sample injection volume was 10 μL. Data were collected using a DAD detector in the wavelength range of 190 to 400 nm, with an interval of 2 nm.

2.6. Chemometric analysis

The data point recorded in each chromatographic run was a function of retention time and absorption wavelength. The spectral data (190–400 nm) of each sample were collected using a DAD detector, resulting in a three-way data array (retention time × absorption wavelength × sample) for multiple samples. According to the trilinear model, each element (x_{ijk}) in the three-way data array ($\underline{\mathbf{X}}$) with a size of $I \times J \times K$ can be expressed using the following equation:

$$x_{ijk} = \sum_{n=1}^{N} a_{in} b_{jn} c_{kn} + e_{ijk}, i = 1, 2, \cdots, I; j = 1, 2, \cdots, J; k = 1, 2, \cdots, K \tag{5}$$

where, a_{in}, b_{jn}, and c_{kn} represent the elements of chromatographic matrix **A** ($I \times N$), spectral matrix **B** ($J \times N$), and relative concentration matrix **C** ($K \times N$), respectively. The term 'e_{ijk}' represents the element of the three-way residual matrix ($\underline{\mathbf{E}}$). N represents the total number of selected factors, including target analytes, unknown interferents, instrument noise, retention time shift, and baseline shift.

The HPLC data, originally in *.csv* format, were converted into MATLAB m-files (*.m*) using a custom-developed program. These files were subsequently stacked along the third dimension to form a three-dimensional data array. Based on its "second-order advantage", the alternating trilinear decomposition-assisted multivariate curve resolution (ATLD-MCR) algorithm was used to obtain the augmented chromatographic matrix **A**, spectral matrix **B**, and relative concentration matrix **C**. The ATLD-MCR algorithm initiates with the alternating trilinear decomposition (ATLD) as its foundational approach, subsequently integrating multivariate curve resolution (MCR) coupled with a least squares optimization strategy to refine the analytical outcomes. It is advantageous due to its insensitivity to several factors, ability to overcome nonlinear challenges, and fast convergence. The codes of ATLD-MCR algorithm were referenced to the Supporting Information and the original literature(Wang, Wu, et al., 2019). All computational analyses were performed using MATLAB software, executed on a robust platform equipped with a 13th Generation Intel(R) Core(TM) i7-13700F processor, 16 GB of RAM, and the Windows 11 operating system.

3. Results and discussion

3.1. Partition behaviors of flavonoids in ATPS

The phase equilibrium of a two-phase aqueous system is represented by a binodal curve phase diagram, which displays the content relationship of each component when forming an ATPS, as shown in Fig. 2. The fitting results of bimodal curves using Merchuk's equation are shown in Table S5. The binodal curve delineates the boundary between two-phase and single-phase regions, with the points on the curve representing the critical phase-forming points. The area below the binodal curve represents a single-phase zone, while the area above it represents a two-phase zone. The closer the curve is to the coordinate origin, the stronger the phase-forming ability.

The formation of two phases is guided by the difference in the binding ability between the formed components and water, and the hydrophobicity strength of the components directly determines their ability to form ATPS(Mashhaditafreshi & Haghtalab, 2024). Generally, a larger logKow value indicates that the component is more hydrophobic (Li et al., 2022). The logKow values of each component namely, betaine, D-glucose, D-sorbitol, xylitol, NPA, and IPA in the DES/alcohol ATPS were observed to be −4.93, −2.89, −3.01, −2.56, 0.35, and 0.28 respectively. Based on these logKow values, the hydrophobicity difference between NPA and DES was observed to be greater than that of IPA. When two short-chain alcohols, NPA and IPA, combine with DES to form an ATPS, the phase-forming ability of NPA is enhanced compared to that of IPA.

In addition, the hydrogen bond acceptors of the three DESs are betaine, resulting in a significant difference in hydrophobicity between the hydrogen bond donors and short-chain alcohols, leading to the variations in phase forming ability. Furthermore, betaine has the smallest logKow value, so its impact on ATPS formation was greater than that of D-glucose, D-sorbitol, and xylitol. When three DESs namely, Bet-Glu, Bet-Sor, and Bet-Xyl are used to construct ATPS using NPA, the phase-forming ability follows the order of Bet-Glu > Bet-Sor > Bet-Xyl. Although the logKow value of D-glucose is higher than that of D-sorbitol, Bet-Glu demonstrated a greater phase-forming ability than Bet-Sor, which may be attributed to the difference in the molar ratio between the hydrogen bond donors and acceptors. In summary, the various types and synthesis ratios of DESs using short-chain alcohols results in variation in hydrophobicity, affecting their ability to form ATPSs.

3.1.1. Selection of types of DESs and alcohols for optimal partitioning of flavonoids

In this study, quercetin was used as a representative of flavonoids to investigate the phase formation behavior of six types of DES-based ATPSs based on its extraction yield. The construction methods for the six types of ATPSs are shown in the Table 3S. As illustrated in Fig. 2(d), for the same DES, the extraction efficiency of ATPSs using NPA was observed to be higher than that of IPA. Furthermore, for the same alcohol, the extraction efficiency of ATPSs using Bet-Sor achieved the highest value of 81.5 %. Therefore, Bet-Sor/NPA ATPS was selected to extract flavonoids from Acacia honey samples for subsequent experiments. Consequently, the Bet-Sor/NPA ATPS was chosen as the optimal medium for the extraction of flavonoids from Acacia honey samples for subsequent experimental procedures.

3.1.2. Effect of parameters for optimal partitioning of flavonoids

Initially, as depicted in the phase diagram for the Bet-Sor/NPA ATPS presented in Fig. 2(b), it is evident that Bet-Sor and NPA are capable of forming a stable ATPS across a wide range of concentrations. The influence of Bet-Sor concentration on the flavonoid extraction yield was systematically examined by adjusting the Bet-Sor concentration within a

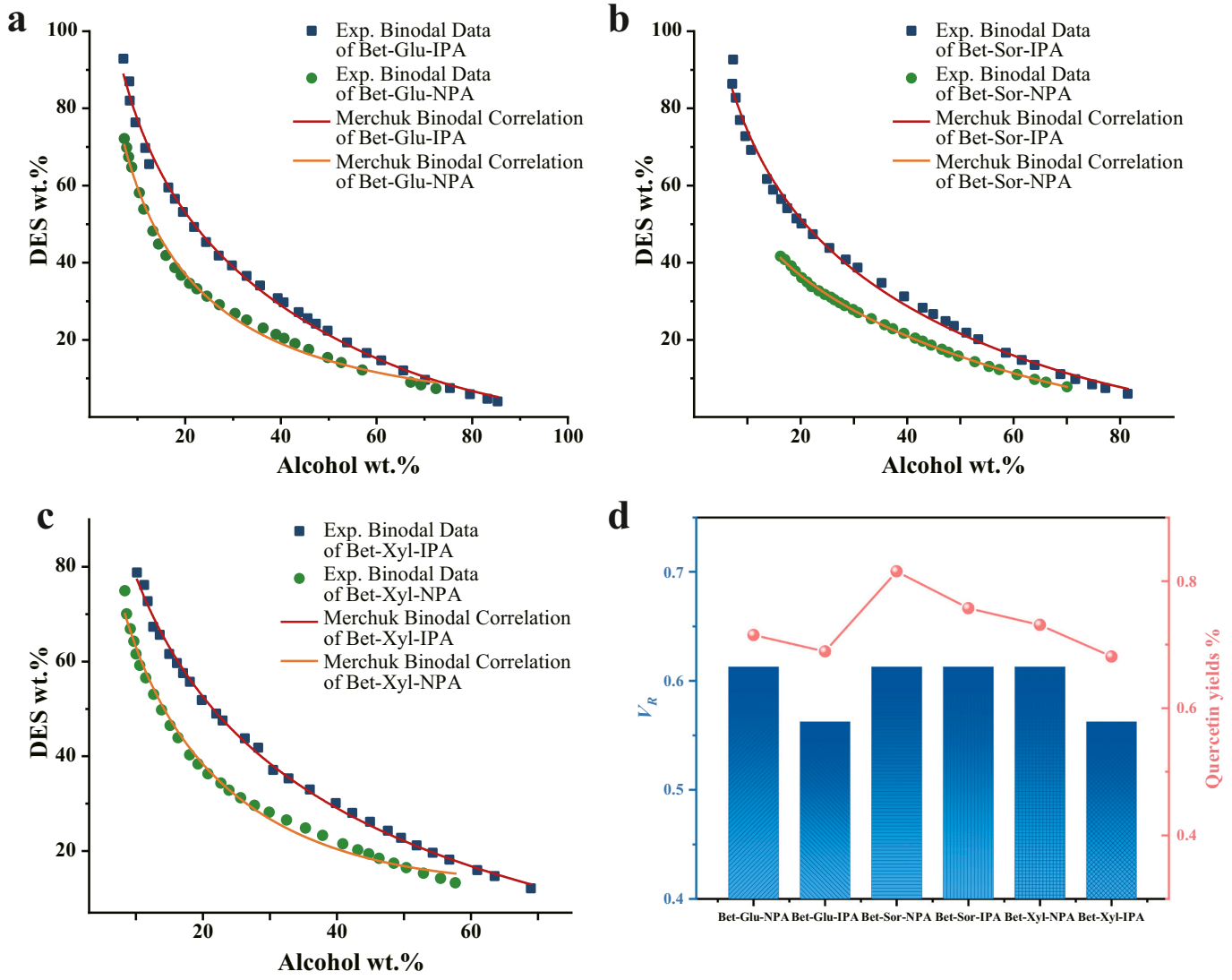

Fig. 2. Phase diagrams at room temperature and atmospheric pressure of DES-based ATPSs composed of (a) Bet-Glu + Alcohol + H_2O, (b) Bet-Xyl + Alcohol + H_2O, (c) Bet-Sor + Alcohol + H_2O, and (d) extraction results of six types of DESs and alcohols.

range of 40 % to 64 % (*w*/w), with the NPA concentration held constant at 30 % (w/w). Approximately 0.1 mL of quercetin working standard solution was added to the system, and sonicated at 45 °C for 20 min, then allowed to stand at room temperature for 30 min. As demonstrated in Fig. 3(a), for Bet-Sor/NPA ATPS, the extraction yield of quercetin exhibited an increasing trend with an increase in DES concentration. However, this trend stabilized when the DES concentration in the system exceeded 46 %. The rationale for this observation could be attributed to enhanced hydration with increased DES concentration, leading to the transfer of water from the top phase to the bottom phase. The increasing hydrophobicity of the top phase facilitated the extraction of quercetin (Yin et al., 2022). With a continuous increase in the Bet-Sor concentration, the phase transfer of water decreased, and the extraction yield of quercetin increased gradually until an equilibrium was achieved. Therefore, the optimal DES concentration for the extraction of quercetin from Bet-Sor/NPA ATPS was determined to be 46 %, with a V_R value of 0.67 and an extraction yield of 85.50 %.

Secondly, the mass fraction of Bet-Sor in the ATPS system was maintained constant at 46 %, and the impact of NPA concentration (24–48 wt%) on the extraction of quercetin was investigated. The effect of varying the NPA concentration on the extraction yield of quercetin in the Bet-Sor/NPA ATPS is illustrated in Fig. 3(b). The extraction yield of quercetin increased with NPA concentration, reaching its maximum at an NPA concentration of 42 %. At lower NPA concentrations, the separation between the two phases was minimal, resulting in a smaller volume of the top phase and subsequently lower extraction yields of quercetin. This phenomenon can be ascribed to the restricted phase separation and the diminished volume of the top phase. With an escalation in the NPA concentration, the top phase volume concurrently expanded, while the bottom phase volume contracted, manifesting a direct linear correlation between the phase ratios of the ATPSs and the NPA concentration. When the concentration of NPA in the system was comparatively low, the volume of the top phase was observed to be less than the quantity of NPA introduced. This observation implies that NPA was also distributed within the bottom phase, thereby diminishing the efficiency of phase separation and complicating the extraction of quercetin from the top phase. As shown in the binodal curve phase diagram of Bet-Sor/NPA ATPS (Fig. 2(b)), the phase-forming ability of the system improved with a constant DES concentration and increased the NPA concentration. As the NPA concentration increased, the degree of separation and the gradient force of the two phases increased, leading to a higher extraction yield of quercetin from the top phase. However, when the NPA concentration exceeded 42 %, the continuous increase in the top phase volume resulted in a dilution of quercetin, causing a decline in

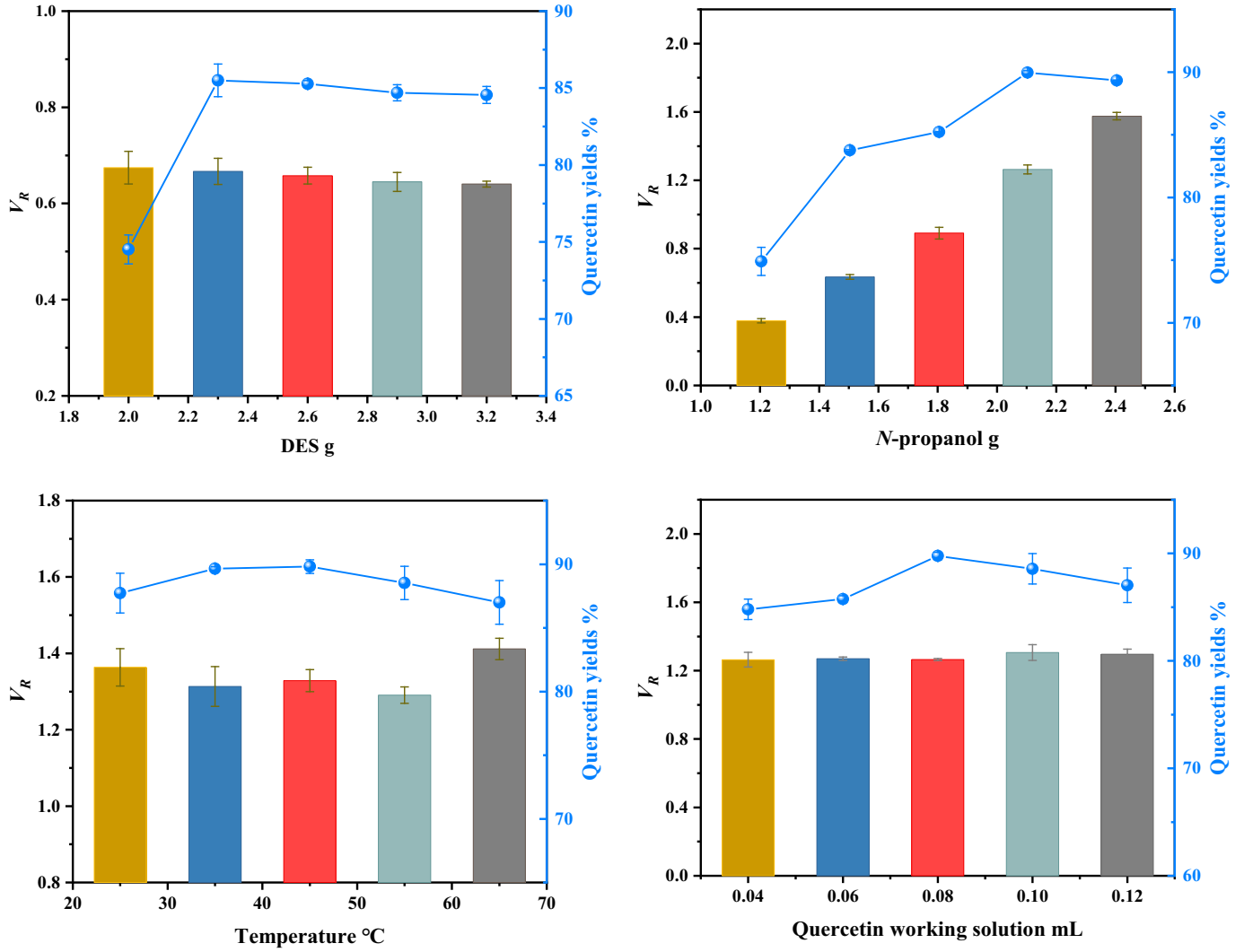

Fig. 3. The effect of single factor experiments on extraction results of quercetin. (a) DES content, (b) *N*-propanol content, (c) temperature, and (d) quercetin addition amount. Line + symbol represents the extraction yields of quercetin, while column represents the phase ratio of DES-based ATPSs.

extraction yield. Therefore, the optimal NPA concentration for the Bet-Sor/NPA ATPS extraction of quercetin was determined to be 42 %, with a V_R value of 1.26 and an extraction yield of 89.97 %.

Thirdly, the effect of temperature on the extraction yield of quercetin was investigated in this study. As shown in Fig. 3(c), temperature had a noticeable but not significant impact on the extraction of quercetin. The extraction yield of quercetin initially increased and then decreased with a change in temperature. The maximum extraction efficiency was observed at 45 °C, achieving an extraction yield of 89.82 %.

Finally, the impact of the volume of quercetin working standard solution on extraction yield was investigated (Fig. 3(d)). It was observed that when the volume was less than 0.08 mL, the extraction yield increased with an increase in the volume of quercetin. However, when the volume exceeded 0.08 mL, the extraction yield decreased with a further increase in quercetin volume. As the quercetin volume increased, the extraction yields gradually reached saturation, and with further increases, the extraction yield indicated a downward trend. Therefore, the optimal volume of quercetin working standard solution was determined to be 0.08 mL, corresponding to a quercetin mass of 13.06 μg in the Bet-Sor/NPA ATPS. Under these extraction conditions, the extraction yield was 89.76 %, with a V_R value of 1.27.

The results from the single-factor experimental analysis can be encapsulated in the following summary: the optimal concentrations of Bet-Sor and NPA concentration, optimal temperature and volumes of working standard solution were determined to be 46 % (*w*/w), 42 % (w/w), 45 °C, and 0.08 mL, respectively. The impact of varying levels on the extraction yield of quercetin can be evaluated through the computation of the mean square error for each single-factor experiment, as delineated by the formula provided below:

$$R = \sqrt{\frac{\sum_{i=1}^{n}(Y_i - \overline{Y})^2}{n-1}} \tag{6}$$

where Y_i (mg/g) denotes the extraction yields of quercetin at each specific level within the single-factor experiment. The term $\overline{Y}$ represents the mean value of all Y_i values, while n signifies the total number of levels examined in each single-factor experiment.

The mean square errors derived from the four individual single-factor experiments, pertaining to the concentrations of Bet-Sor and NPA, the temperature, and the additional volumes of the quercetin working standard solution, were determined to be 4.70, 6.05, 1.21, and 1.90, respectively. That is, the individual impact of each single factor on the extraction yield of quercetin is delineated as follows: NPA concentration>Bet-Sor concentration>quercetin addition volumes>temperature. Therefore, the temperature was set at the optimal level of 45 °C, while the concentrations of NPA at 36 %, 42 %, and 48 %, Bet-Sor at 40 %, 46 %, and 52 %, and the additional volumes of the

quercetin working standard solution at 0.06 mL, 0.08 mL, and 0.10 mL were utilized as variables. These parameters were employed to refine the ATPS extraction conditions through the application of response surface methodology in the subsequent experiments.

3.1.3. Response surface methodology optimization of ATPS extraction conditions

As shown in Table S4, 17 experiment results ranging from 86.68 % to 97.17 % were obtained based on the Box-Behnken design. Then, the experiment data was organized and analyzed by using Design-Expert software (Table 1), establishing the following regression model equation:

$$Y = 96.41 - 1.21X_1 + 2.63X_2 + 1.15X_3 + 0.22X_1X_2 - 0.28X_1X_3 - 2.62X_2X_3 - 1.73X_1^2 - 3.46X_2^2 - 1.49X_1^2X_2 - 1.13X_1^2X_3 \quad (7)$$

where Y is the response variable, and X_1, X_2, and X_3 are the test variables.

The RSM model expressed in Eq. (7) has a determination coefficient (R^2) of 0.9861 and a signal-to-noise ratio (with adequate precision) of 22.45, indicating that the model has high statistical significance, and fits well with the experimental data. The RSM model, as expressed in Eq. (7), boasts a determination coefficient (R^2) of 0.9861 and a signal-to-noise ratio (with adequate precision) of 22.45. These metrics underscore the model's robust statistical relevance and its excellent alignment with the experimental data. As shown in Table 1, *P*-values that fall below the threshold of 0.01 signify that the model terms are statistically significant. Specifically, the terms X_1, X_2, X_3, the interaction X_2X_3, and the squared terms X_1^2, X_2^2, along with the interaction of squared terms $X_1^2X_2$, $X_1^2X_3$, are identified as significant influencing factors within the model. By comparison with Bet-Sor content (X_3) and addition volume of quercetin working standard solution (X_1), it was found that the NPA concentration (X_2) is the paramount variable influencing the extraction yields of quercetin and the optimization of extraction conditions. The *F*-value for the lack of fit, which stands at 0.43, suggests that the lack of fit is negligible in comparison to the pure error. Consequently, the optimized model demonstrates statistical significance, coupled with persuasive accuracy and reliability.

Utilizing the regression equation derived from the analysis, three-dimensional (3-D) plots were constructed to visually represent the interplay between the parameters. These plots, as presented in Fig. 4, demonstrate how the interactions among different factors influence the extraction yield of quercetin. The interplay between the concentration of NPA and the additional volumes of quercetin exerts the most pronounced effect on the extraction yield of quercetin, as depicted in Fig. 4 (a). The response surface exhibits an upward convexity, indicating a peak extraction yield within the experimental range. As shown in Fig. 4 (c), the interaction of Bet-Sor concentration and additional volumes of quercetin has a secondary impact on the extraction yield, while the impact of NPA concentration and Bet-Sor concentration on quercetin extraction was not observed to be significant (Fig. 4(b)). The maximum extraction yield of quercetin can reach 96.49 %. The optimal DES-based aqueous two-phase extraction conditions are 45 °C, 45 % (*w*/w), 40 % (w/w), and 0.06 mL corresponding to temperature, NPA concentration, Bet-Sor concentration, and additional volume of quercetin working standard solution, respectively. Therefore, these specific conditions were deemed optimal for the extraction of flavonoids from Acacia honey, and were subsequently adopted for the HPLC-DAD experiments.

Table 1
The anova for quadratic model of the response surface experiments.

Source	Sum of squares	D_f	Mean square	*F*-value	*P*-value	Significant [a]
Model	143.96	10	14.40	42.67	< 0.0001	**
X_1- quercetin addition amount	11.76	1	11.76	34.86	0.0010	**
X_2- *n*-propanol	27.62	1	27.62	81.86	0.0001	**
X_3- DES	5.27	1	5.27	15.61	0.0075	**
X_1X_2	0.1936	1	0.1936	0.5739	0.4774	
X_1X_3	0.3025	1	0.3025	0.8967	0.3802	
X_2X_3	27.41	1	27.41	81.24	0.0001	**
X_1^2	12.68	1	12.68	37.59	0.0009	**
X_2^2	50.56	1	50.56	149.88	< 0.0001	**
$X_1^2X_2$	4.46	1	4.46	13.21	0.0109	*
$X_1^2X_3$	2.54	1	2.54	7.54	0.0335	*
Residual	2.02	6	0.3373			
Lack of Fit	0.3565	2	0.1783	0.4276	0.6787	
Pure Error	1.67	4	0.4169			
Cor Total	145.98	16				

a ** $p < 0.01$, * $p < 0.05$.

3.2. Quantitative determination of flavonoids in acacia honey by ATLD-MCR

In this study, the optimized DES-based ATPS extraction method, combined with chemometric-enhanced HPLC-DAD, was employed for the simultaneous extraction and detection of flavonoids in Acacia honey. Employing straightforward isocratic elution chromatographic conditions, the chromatograms for five distinct flavonoids namely, myricetin, fisetin, quercetin, hesperidin, and kaempferol, along with an unidentified overlapping interference, were successfully captured and are presented in Fig. 5. Furthermore, a noticeable degree of retention time drift was observed in the chromatographic profiles of the target analytes when comparing the calibration samples with those of the Acacia honey samples. To address this, chemometric multivariate calibration analysis, specifically employing a second-order calibration algorithm known as ATLD-MCR, can be effectively utilized to process the HPLC-DAD data.

3.2.1. Construction and validation of the ATLD-MCR model

As detailed in Section 2.2 and Table S1, the ATLD-MCR model was constructed utilizing six calibration samples, and highly satisfactory results yielded with determination coefficients (R^2) exceeding 0.9990 for each of the five analytes. Firstly, the accuracy of ATLD-MCR model was established. Three interference-free validation standards were prepared by mixing their individual standard solutions. The resolved augmented retention time channel, chromatogram (A), spectral (B), and concentration profile (C), have been successfully obtained and are illustrated in Fig. S3, Fig. S4, and Fig. S5, respectively. Table 2 displays the quantitative results of the five flavonoids predicted using the ATLD-MCR method. Chromatograms, spectra, and relative concentration information of five flavonoids were obtained in the presence of co-elution and retention time drift. The average recovery was observed between 70.06 % and 92.11 %, with a standard deviation of less than 1.52 %. The recoveries of flavonoids, excluding myricetin, were observed to be greater than 85 %.

The low recovery of myricetin may be attributed to the following reasons: Firstly, the phenolic hydroxyl groups in its molecular structure led to increased water solubility and transfer to the DES phase, resulting in decreased extraction yield in the NPA phase. Secondly, the chromatographic retention time of myricetin is relatively short and greatly influenced by solvent and instrument noise. Moreover, honey sample has a complex background matrix and is a supersaturated solution of sugar, which mainly includes 38 % of fructose and 31 % of glucose, containing also minerals, proteins, free amino acids, enzymes and vitamins(Zhang et al., 2024; Zhang, Gu, Liu, Qing, & Nie, 2023). Considering the complexity of the background and simple operating conditions, the results obtained were satisfactory and met the determination requirements. These results provided further evidence for the excellent performance of the ATLD-MCR method. Therefore, in the following section, the resolution capability of this procedure in

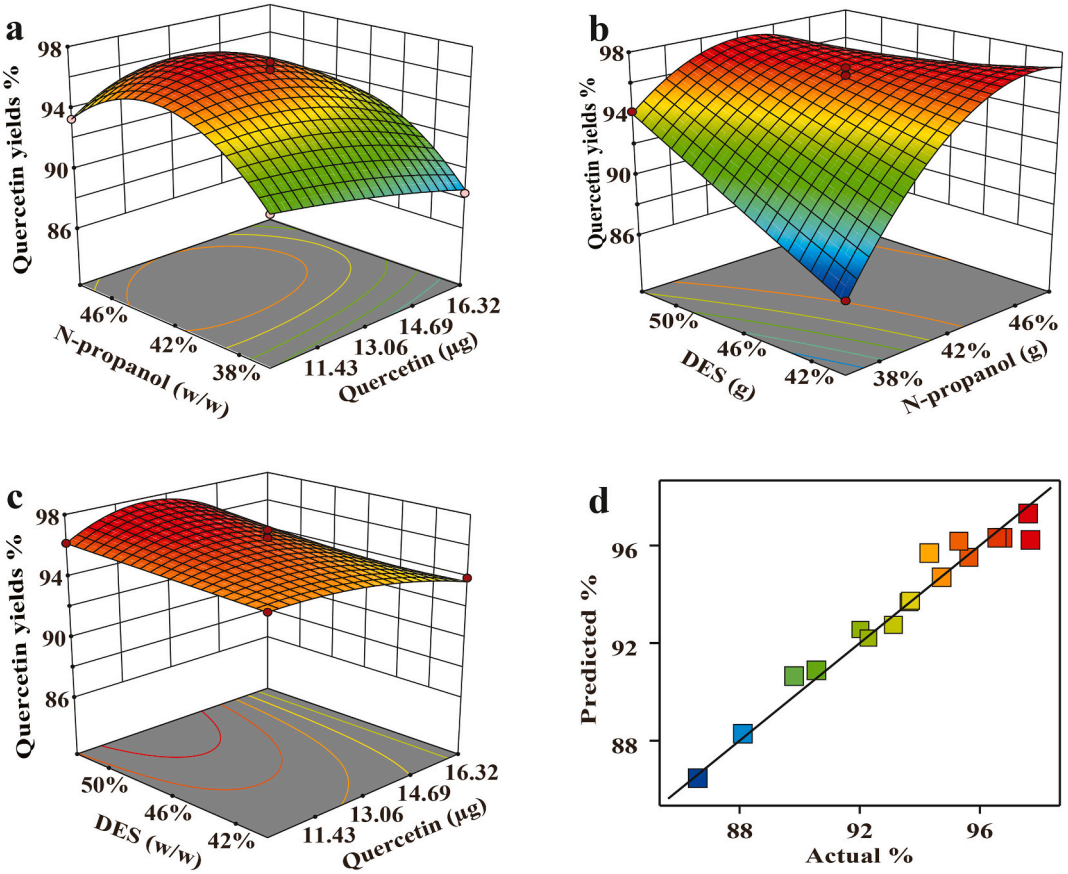

Fig. 4. The interaction effects on extraction yields of quercetin. (a) *N*-propanol content and the adding volume of quercetin working solution, (b) DES content and *N*-propanol content, (c) DES content and the adding volume of quercetin working solution, and (d) predicted values vs. Actual values.

quantifying five flavonoids in more complex honey systems was further investigated.

3.2.2. *Quantification in real honey samples*

The effect of additional amount of honey on the extraction efficiency of flavonoids is illustrated in Table S6. The content of quercetin in Acacia honey was not detected as it was below the detection limit. Additionally, the extraction yield of the other three flavonoids, excluding myricetin, showed a downward trend with an increase in the amount of honey added. Therefore, the minimum amount of honey was selected for the subsequent experiments. As the amount of honey pretreated solution added was smaller and myricetin was not detected, an optimal volume of 0.6 mL was selected for the experiment.

Fig. 5(a) depicts the chromatograms (A), spectra (B), and concentration profiles (C) of myricetin and fisetin obtained using ATLD-MCR. The chromatographic profiles of myricetin and fisetin overlapped, significant instrument noise and baseline drift were observed. The two factors used to fit the background are indicated by solid blue and cyan lines, as shown in Fig. 5(a). The quantitative results of analytes, including predicted concentration, spiked recoveries and standard deviations in Acacia honey using ATLD-MCR are presented in Table 2. The predicted concentration for flavonoids namely myricetin, fisetin, hesperidin, and kaempferol, excluding quercetin (not detected) in the Acacia honey samples were 0.15 μg/g, 2.65 μg/g, 1.15 μg/g, and 1.28 μg/g, respectively as shown in Table 2. Among the target flavonoids, fisetin exhibited the highest concentration, contrasting with myricetin, which displayed the lowest concentration. The average recoveries for the five flavonoids ranged between 74.0 % to 86.6 %, with a standard deviation of less than 2.2 %. The relatively poor results for myricetin and fisetin may be attributed to lower extraction efficiency, a complex background matrix, and instrument noise. However, the overall detection requirements were met, providing a green, efficient, and accurate method for the extraction and determination of flavonoids in honey. Furthermore, the quantitative results obtained in this study are in the same order of magnitude compared to reported methods(Zhang et al., 2013). However, the pretreatment method was simpler and more effective. Differences in the content of some flavonoids may be due to variations in sample preparation methods. There are also differences in the types and contents of flavonoids in various honey samples, which can be attributed to factors such as origin, variety, and climate.

3.3. *GREEnness assessment of the developed method*

Various researchers have proposed greenness assessment methods for analytical procedures, such as the AES (Analytical Eco-Scale) proposed by Gałuszka et al.(Gałuszka, Migaszewski, Konieczka, & Namieśnik, 2012) and the GAPI (Green Analytical Procedure Index) tool proposed by Płotka-Wasylka(Płotka-Wasylka, 2018). However, these methods have certain disadvantages, such as inconsistent and incomplete selection of evaluation criteria, hence it is important to choose a more comprehensive and authoritative evaluation criterion. In 2020, Pena-Pereira et al.(Pena-Pereira, Wojnowski, & Tobiszewski, 2020). proposed a relatively comprehensive assessment approach for analytical greenness (AGREE), which provided 12 evaluation criteria covering various dimensions. A freely available software was developed to calculate the greenness of an analytical method and generate a pictogram indicating the final score based on multiple input values. Using the AGREE calculator, the greenness of this method was evaluated and compared with earlier studies. As shown in Table 3, this method has the highest green index, covering three aspects. Firstly, the synthetic raw

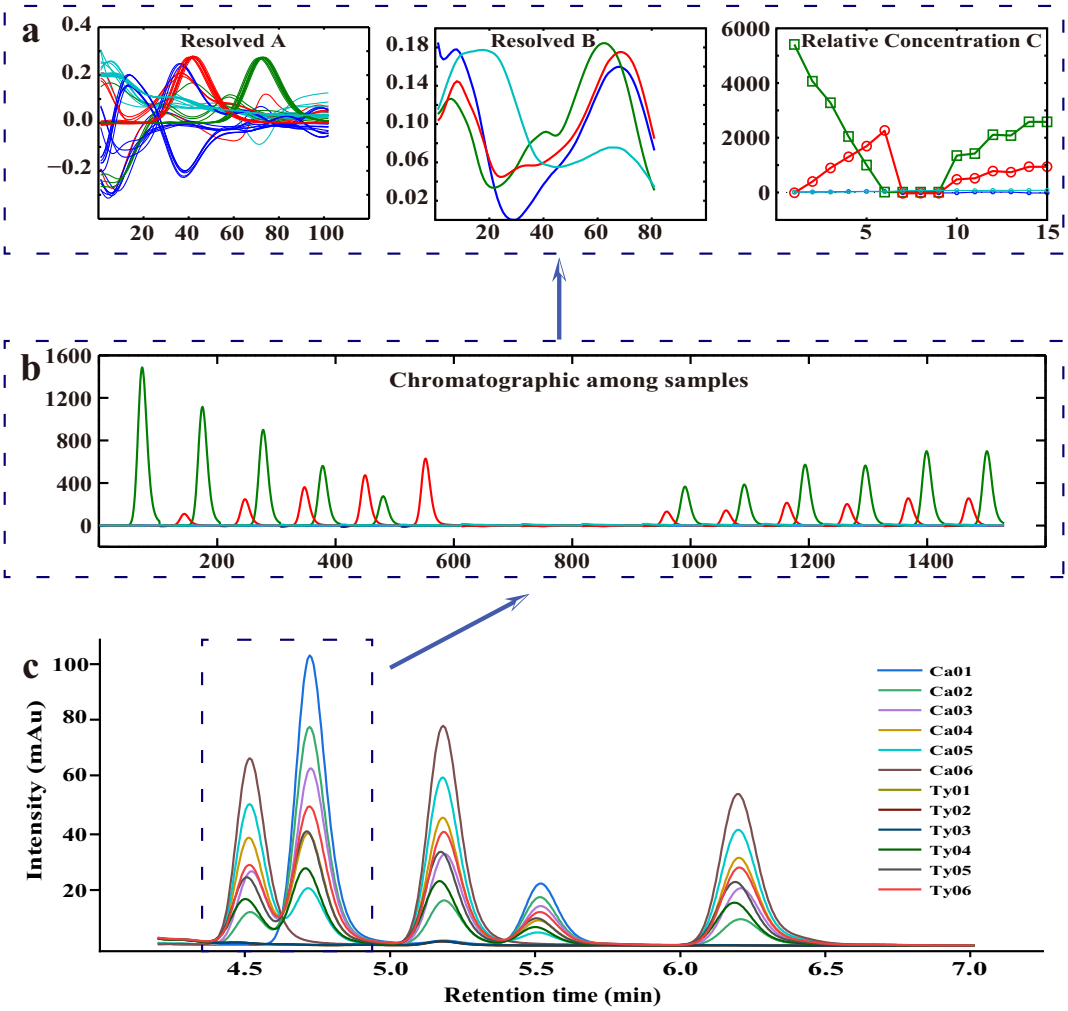

Fig. 5. The results resolved by ATLD-MCR algorithm for myricetin and fisetin (a, b) and the stacked chromatograms (c) of calibration and Acacia honey samples.

Table 2
The quantitative results of analytes in mixed standard solutions and acacia honeys predicted by the ATLD-MCR method.

	Myricetin	Fisetin	Quercetin	Hesperidin	Kaempferol
Standard solutions					
Real concentration (μg/mL)	8.53	16.55	8.74	5.44	5.44
PS01 (μg/mL)	5.87	14.04	7.46	4.86	4.90
PS02 (μg/mL)	5.99	14.31	7.62	4.93	5.00
PS03 (μg/mL)	6.06	14.66	7.83	5.06	5.13
Recovery (%)	70.06	86.62	87.36	91.01	92.11
RSD (%)	0.80	1.31	1.47	1.37	1.52
Acacia honeys					
Predicted contents (μg/g)	0.15 ± 0.090	2.65 ± 0.021	-[a]	1.15 ± 0.065	1.28 ± 0.005
Sa01 (μg/mL)	5.22	10.13	5.35	3.33	3.33
Sa02 (μg/mL)	7.83	15.20	8.03	4.99	4.99
Sa03 (μg/mL)	9.40	18.24	9.63	5.99	5.99
Recovery (%)	74.00	80.40	83.69	85.85	86.63
RSD (%)	0.70	0.82	2.20	1.48	0.69

[a] - denotes not detected.

materials of DES were green, environmental friendly, inexpensive, and easily available, with characteristics such as biodegradability and good biocompatibility. Secondly, this method utilized DES to construct a novel ATPS, which shortened the extraction time and reduced experimental costs compared to solid-phase extraction. Additionally, this method involved simpler stages compared to traditional liquid-phase extraction, adopted DES-based aqueous phase extraction procedure that to the principles of green chemistry. Finally, the "second-order advantage" of the multivariate calibration method in chemometrics was employed, which utilizes "mathematical chromatography" to partially replace traditional chromatography or mass spectrometry. Rapid and accurate qualitative and quantitative results of the target analytes were achieved, further enhancing the greenness of the experimental method. Therefore, the method proposed in this study has characteristics of high greenness, fewer extraction stages, short extraction and analysis time, high recoveries, and good reproducibility. It can be applied in the extraction and quantitative analysis of active components in honey processing products, agricultural products, traditional Chinese medicines, and other related fields.

4. Conclusions

In this study, a green analytical method was developed combining deep eutectic solvent(DES)-based aqueous two-phase systems (ATPSs) with chemometrics for simultaneous extraction and detection of flavonoids in honey. The composition and synthesis ratio of DESs influenced their hydrophobic interaction with alcohols, which in turn affects their capacity to establish ATPSs. The Bet-Sor/NPA ATPS was identified as the most effective extraction system for the target analytes, attaining a

Table 3
Methods for extraction and analysis of flavonoids from plant materials.

Sample	Analytes	Extraction method	Extraction solvent	Analysis method	Analysis time/ min	Recovery/ %	Greenness assessment	Ref
Flos sophorae immaturus	Rutin, nicotiflorin, narcissoside, kaempferol, isorhamnetin, and quercetin	Ultrasound-assisted extraction	Ethanol	HPLC-UV	50	97.7–99.4		(Fan, Yang, Zhang, Li, & Bai, 2020)
Pi-nus massoniana Lamb	Total flavonoids	Microwave-assisted extraction	Ionic liquids	UV–visible spectrophotometry	/	/		(Zuo, Ao, & Guo, 2020)
Aegle marmelos	Marmelosin	Microwave-assisted extraction	Ethanol-water solution	HPLC-UV	10	/		(Sonar & Rathod, 2020)
Polygonum aviculare leaves	Myricitrin, 3''-O-galloyl-myricitrin, quercitrin, and avicularin	Ultrasound-assisted extraction	Deep eutectic solvents	HPLC-DAD	40	/		(Wu, Chen, Li, Wang, & Zhang, 2021)
Selaginella uncinata	Amentoflavone and robustaflavone	Microwave-assisted extraction	Deep eutectic solvents	UPLC-Q-TOF-MS	40	1.17–1.58		(Qin et al., 2022)
Acacia honey	Myricetin, fisetin, quercetin, hesperidin, and kaempferol	Aqueous two-phase extraction	*N*-propanol/ Deep eutectic solvents	HPLC-DAD	6.5	74.0–86.6		This work

maximum extraction efficiency of 81.5 %. Through conducted single-factor experiments and subsequent optimization via response surface methodology, the optimal extraction parameters were established as follows: a temperature of 45 °C, NPA concentration at 45 % (*w*/w), Bet-Sor concentration at 40 % (w/w), and a volume of 0.06 mL for the quercetin working standard solution, respectively. Under these optimized DES-based ATPS extraction conditions, the extraction process yielded a remarkable maximum extraction efficiency for quercetin, reaching 96.49 %. The optimized DES-based ATPS extraction method, combined with chemometric-enhanced HPLC-DAD, was employed for the simultaneous extraction and detection of five flavonoids in Acacia honey. The average recoveries observed ranged from 74.0 % to 86.6 %, with a commendably low standard deviation, falling below 2.2 %. Finally, employing an AGREE calculator, the green chemistry metrics of this study were evaluated and compared with those from prior studies. The findings indicated that our method not only adhered to the tenets of green chemistry but also garnered a notably high green index, amounting to 0.71. This innovative DES-based ATPS stands out as an eco-friendly approach, perfectly suited for the extraction of bioactive constituents from botanical sources. Furthermore, the combination of DES-based ATPS and chemometrics paves the way for a flexible and sustainable for efficiently extracting and accurately analyzing various biomolecules in complex samples.

CRediT authorship contribution statement

Xiao-Hua Zhang: Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Ming-Xuan Li:** Software, Investigation, Formal analysis. **Shi-Yu Li:** Software, Methodology, Formal analysis. **Jie Su:** Software, Methodology, Formal analysis. **Li-Ying Wei:** Methodology, Formal analysis, Data curation. **Yan-Ting Yuan:** Resources, Formal analysis, Data curation. **Peng-Hua Shu:** Visualization, Validation. **Kewen Tang:** Writing – review & editing, Supervision, Resources, Methodology, Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101932.

Data availability

Data will be made available on request.

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