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Analytical Methods

Aqueous two-phase systems coupled with chemometrics-enhanced HPLC-DAD for simultaneous extraction and determination of flavonoids in honey

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ABSTRACT

In this study, an accurate, rapid, green, and environment friendly method for the extraction and quantitative analysis of flavonoids in honey was established by using the aqueous two-phase extraction combined with the chemometrics-assisted HPLC-DAD. The first purpose of this study was to extract seven flavonoids in five different types of honey using alcohol/salt aqueous two-phase system (ATPS). The system with 2.82 mL sodium citrate (30%), 1.58 mL water, and 3.10 mL isopropanol, showed the highest flavonoids extraction yields in the top phase (87.66–101.50%). Additionally, the three-way array of honey samples based on HPLC-DAD was decomposed mathematically by the alternating trilinear decomposition (ATLD) algorithm to obtain reasonable chromatograms, spectra, and concentration profiles for each analyte. Compared with the traditional solid-phase extraction method, the ATPS-ATLD-based method showed satisfactory spiked recoveries, lower limit of detection, and higher sensitivity, further verifying its accuracy and stability.

1. Introduction

Honey, a natural food product collected from the melliferous plants by honeybees, has high economic value due to its nutritional and health benefits. It contains more than 200 substances, including 65–80% carbohydrates (glucose and fructose), 18% water, and traces of protein, phenolic acids, flavonoids, free amino acids, pigments, enzymes, vitamins, alkaloids, and aroma compounds (Maione, Barbosa, & Barbosa, 2019; Tsagkaris, et al., 2021). Among them, Flavonoids are the essential active components in honey. Clinical studies have found that flavonoids play a significant role in preventing, protecting, reducing, and treating numerous chronic diseases (Đogo Mračević, et al., 2020; Jucá, et al., 2020; Sammani, Clavijo, & Cerdà, 2021). Generally, the types and contents of flavonoids depend on the plant and geographic origin of honey and the climatic conditions of the region (Nascimento, et al., 2018; Se, et al., 2019; Stanek & Jasicka-Misiak, 2018). As the common and naturally occurring secondary metabolites in honey, flavonoids can be used as effective quality indicators of honey (Biluca, et al., 2020; Rusko, et al., 2021; Vasic, et al., 2019). Thus, the efficient extraction and accurate quantification of flavonoids in honey may provide a powerful theoretical reference and guidance for the source identification and quality control of honey, which is of great significance to promote the healthy development of the honey industry.

The analytical methods for determining flavonoids in natural products usually include the extraction of the analytes using selective pretreatment methods combined with chromatographic analysis. For example, Nascimento et al. analyzed total phenolics and flavonoids as well as individual composition using liquid-liquid extraction (LLE) in combination with high-performance liquid chromatography (HPLC) (Nascimento, et al., 2018). Stanek et al. established a rapid method based on column chromatography (CC) and high performance thin layer chromatography (HPTLC) for the qualification and quantification of

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phenolic compounds and abscisic acid in honey samples (Stanek, Kafarski, & Jasicka-Misiak, 2019). Teodora et al. performed a study using a solid phase extraction (SPE) method combined with ultra-high-performance liquid chromatography diode array detector electrospray ionisation mass spectrometry (UHPLC-DAD-ESI/MS) to investigate the phenolic compounds profile and bioactive properties of different honey types from Romanian flora in order to develop reliable tools for honey floral origin (Teodora & Elisabeta-lrina, 2019). However, for these traditional extraction combined with chromatography-based methods, several issues may arise due to the complex background matrix of honey, including complex pretreatment steps, retention time and baseline drift, co-eluted peaks as well as unknown interferences, affecting the accuracy of the results (Zhang, et al., 2020; Zhang, et al., 2023). Thus, it is necessary to develop simple and efficient extraction and quantitative determination methods for the analysis of flavonoids in honey.

The most common extraction methods (such as traditional organic phase extraction methods) have the limitations of prolonged extraction time, low extraction rates, and introduction of a large number of toxic reagents in the extraction process, causing harm to the environment and operators (Medina, et al., 2019; Nascimento, et al., 2018; Wang et al., 2021b). The SPE method is advantageous over the common LLE methods due to the easy removal of interferents but is more expensive and has cumbersome steps (Melekhin, et al., 2021; Pang, et al., 2021). Additionally, the selectivity of SPE might change due to the distribution of analytes during a multi-component analysis. Recently, the aqueous two-phase systems (ATPS), consisting of short-chain alcohols/hydrophilic solvents and inorganic salts, have emerged as a significantly improved LLE method for the extraction of active compounds in the complex systems (Chong, et al., 2020; Enriquez-Ochoa, et al., 2020; Zhao, et al., 2021). The partition behavior of the analytes in ATPS mainly depends on their physical and chemical properties, such as hydrogen bonding, charge, hydrophobicity, etc. By using the ATPS extraction method, it can achieve a high extraction rate and high product purity at the same time, and maintain the biological activity of the molecules (Gao, et al., 2020; Yao, et al., 2022). Compared with the traditional extraction methods, it has the characteristics of mild extraction conditions, small overall volume, fewer self-adjusting factors, and high extraction rate. It has been successfully applied to the separation and purification of proteins, enzymes antibiotics, and other aspects (de França do Rosário, et al., 2019; Lin, et al., 2019; Ran, et al., 2019). However, there are very few reports on ATPS application in the separation and enrichment of active components in honey due to relatively low contents and complex background matrix. Honey background matrix is complex and is a supersaturated solution of sugars, but with a low content of flavonoids. Therefore, compared with other food matrices, efficient sample extraction methods are especially important. In addition, a more efficient sample pretreatment enriches the method selectivity and sensitivity, protects the analytical columns and decreases the matrix effect, allowing the collection of better results. Thus, we tried to use ATPS in this study to remove carbohydrates, proteins, enzymes and other interferents in honey, so as to effectively extract flavonoids.

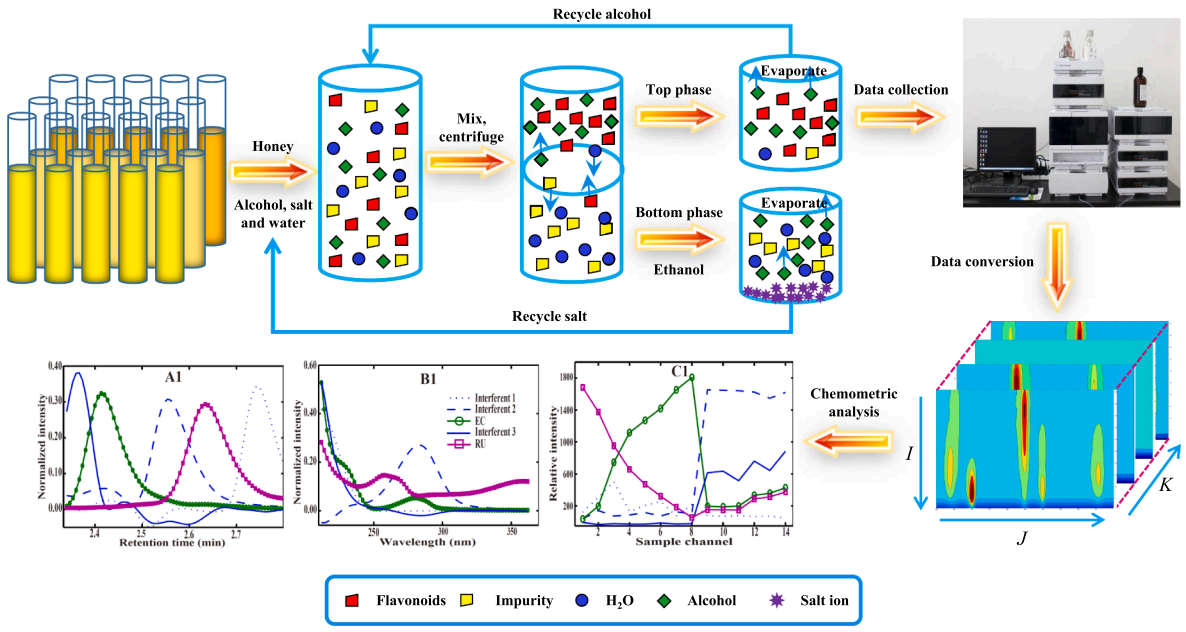
On the other hand, the data arrays obtained from chromatography-based analysis contain not only a lot of useful chemical information, but also the response of unknown interferents, background and/or instrument noise. The effective processing and analysis of these large and complex high-dimensional arrays is required to successful quantitative and qualitative analysis of flavonoids in honey. Recently, the development of multivariate calibration technologies, especially the second-order calibration methods, can well resolve the problem of high-way quantitative and qualitative analysis of target analytes in the complex samples. Under the conditions of unknown interferents coexistence, the "mathematical chromatography" could be used instead of "chromatography or mass spectrometry" to quickly and accurately realize the qualitative and quantitative analysis of the analytes (Olivieri & Escandar, 2019; Zhang, et al., 2021; Zhang, et al., 2020). These methods can also well calibrate the retention time and baseline drifts in the quantitative analysis of hyphenated chromatography, so as to obtain more accurate analysis results (Olivieri & Escandar, 2019; Zhang, Qing, & Wu, 2019). In this study, the second-order calibration method based on the alternating trilinear decomposition (ATLD) algorithm was applied to the qualitative and quantitative analysis of seven flavonoids in five different types of honey. The matrix of honey is extremely complex and contains a variety of unknown sources of interference. However, based on the existing "second-order advantage", this algorithm use the mathematical methods to successfully distinguish the overlapping signals into pure profiles of analytes and interferents, and reasonable chromatographic and spectral profiles of each analyte and accurate concentration predictions can be obtained.

The present study is the first attempt to extract and determine flavonoids in honey by alcohol/salt ATPS combined with chemometrics-enhanced HPLC-DAD. Firstly, the extraction conditions of ATPS were optimized, and the $Na_3C_6H_5O_7$/isopropanol ATPS was selected to fully extract the flavonoids into the top phase, so as to achieve the extraction and enrichment of target analytes in the honey. The bottom phase mainly contains the impurities of honey, such as carbohydrates, proteins, and minerals. Subsequently, the efficient preprocessing technology combined with the chemometrics-assisted HPLC-DAD, an accurate, fast, green, and environment friendly method was established for the extraction and quantitative analysis of flavonoids in honey. Scheme 1 displays an overview flowchart of the proposed method in this work. The results showed that the ATPS-ATLD-based method could well extract flavonoids from honey with high extraction yields, and simultaneously realize the quantitative analysis of seven flavonoids (i.e. epicatechin, rutin, myricetin, fisetin, quercetin, hesperidin, and kaempferol) in five types of honey from different sources. Moreover, this experimental method does not require a large number of toxic reagents, thereby eliminating the problem of residual organic solvents. In a word, the combination of efficient $Na_3C_6H_5O_7$/isopropanol ATPS and chemometrics-assisted HPLC-DAD provided a powerful tool for the simultaneous extraction and determination of flavonoids in honey.

2. Materials and methods

2.1. Reagents and materials

HPLC-grade standards, including epicatechin (EC), rutin (RU), myricetin (MY), fisetin (FI), quercetin (QU), hesperidin (HE), and kaempferol (KA) were purchased from Aladdin Chemistry Co., Ltd (Shanghai, China). HPLC-grade methanol and formic acid were purchased from Fisher Chemical (USA) and Aladdin (China), respectively. The analytical reagents, including ammonium sulfate $(NH_4)_2SO_4$ and sodium citrate ($Na_3C_6H_5O_7$), ethanol, *n*-propanol, isopropanol, sodium hydroxide, disodium hydrogen phosphate, sodium dihydrogen phosphate, and hydrochloric acid were purchased from Sinopharm Chemical Reagent Co., Ltd. Four types of honey from different floral origins (jujube, acacia, clover, and chaste) were supplied by the Henan Zhuoyu Bees Industry Co. Ltd. (Changge, China). Three fragrant nectar was mainly obtained by honeybees collecting the nectar of three kinds of traditional Chinese medicines (Sandalwood, Dalbergia odorifera and Eaglewood), provided by Zhaoqing Jinlongtan Ecological Agriculture Development Co., Ltd. (Zhaoqing, China). Corn syrup was obtained from Shanghai Haoyue Industrial Co., Ltd (Shanghai, China). Three SPE columns, including Poly-Sery PSD solid-phase extraction column (PSD, 250 mg, 6 mL), Poly-Sery HLB solid-phase extraction column (HLB, 200 mg, 6 mL), and Poly-Sery Mixed anion exchange solid-phase extraction column (MAX, 150 mg, 6 mL) were purchased from ANPEL Laboratory Technologies Co., Ltd (Shanghai, China). The Milli-Q integral water purification system (EMD Millipore, Burlington, USA) was used to prepare ultrapure water (with a resistivity of 18.2 mΩ cm at 25 ℃). All other reagents were of analytical grade and used directly without further purification.



Scheme 1. Overview flowchart for the ATPS-ATLD-based method.

2.2. Sampling and preparation

The stock solutions of seven flavonoids were prepared in methanol and stored in a refrigerator at 4 ℃. The concentrations were as follows: EC 2.01×10^6 ng/mL, RU 2.01×10^6 ng/mL, MY 1.93×10^6 ng/mL, FI 2.03×10^6 ng/mL, QU 2.01×10^6 ng/mL, HE 1.98×10^6 ng/mL, and KA, 2.09×10^6 ng/mL. The working solutions of each analyte were prepared by appropriately diluting the stock solutions with mobile phase solutions according to the needs. A total of eight calibration samples were prepared to build a calibration model. Since there were many target analytes in this study, the preparation method of the calibration sample was retained simple. Briefly, different volumes of working solution were poured into a 10.0 mL brown volumetric flask and made up to the mark with the mobile phase solution. In the calibration set, the maximum concentration of each analyte was designed to have a chromatographic response intensity around 20 mAu and was randomly designed within its linear range. In addition, the analytes with similar structures were cross-designed to avoid serious interference (Table S1).

According to the needs of ATPS experiments, the following standard solutions were prepared: ethanol standard solution, *n*-propanol standard solution, and isopropanol standard solution. An appropriate volume of stock solutions of seven flavonoids was poured into a 100.0 mL volumetric flask, diluted to the mark with ethanol, *n*-propanol or isopropanol, and mixed through a vortex to obtain the ethanol standard solution, *n*-propanol standard solution, or isopropanol standard solution, respectively. The contents of the seven flavonoids in the standard solutions were as follows: EC 8.04×10^3 ng/mL, RU 10.05×10^3 ng/mL, MY 5.79×10^3 ng/mL, FI 12.69×10^3 ng/mL, QU 6.53×10^3 ng/mL, HE 3.47×10^3 ng/mL, and KA 8.36×10^3 ng/mL.

An appropriate volume of stock solutions of seven flavonoids was poured into a 5.0 mL volumetric flask, diluted to the mark with corn syrup, and mixed by eddying to simulate the composition of honey. Later, the mixture was sonicated at no higher than 50 ℃ for 20.0 min to ensure that the stock solutions were fully mixed with the corn syrup. The contents of flavonoids in the simulated honey were as follows: EC 40.20×10^3 ng/mL, RU 52.26×10^3 ng/mL, MY 30.88×10^3 ng/mL, FI 73.08×10^3 ng/mL, QU 40.20×10^3 ng/mL, HE 15.84×10^3 ng/mL, and KA 41.80×10^3 ng/mL.

2.3. Aqueous two-phase extraction

2.3.1. Phase diagrams determination

The phase diagram of alcohol-salt was prepared by a turbidity titration method according to the previously described methods (Enriquez-Ochoa, et al., 2020). The aqueous solutions of salt with a known composition (30%), including $(NH_4)_2SO_4$ and $Na_3C_6H_5O_7$ were prepared by weight. Later, the salts were placed in the tubes and titrated with alcohol (ethanol, *n*-propanol or isopropanol) until the mixture became cloudy, corresponding to the transition to the biphasic region. Afterwards, the ultrapure water was added dropwise until the mixture formed a clear solution single-phase region. This process was repeated until enough data was obtained to cover the binodal curve. The composition of the mixtures was determined by weight. All procedures were performed under constant stirring at room temperature.

2.3.2. Partition behaviors of flavonoids in ATPS

In this study, a total of six types of ATPS were applied to verify the partition behavior of flavonoids, including $(NH_4)_2SO_4$/ethanol ATPS, $Na_3C_6H_5O_7$/ethanol ATPS, $(NH_4)_2SO_4$/*n*-propanol ATPS, $Na_3C_6H_5O_7$/*n*-propanol ATPS, $(NH_4)_2SO_4$/isopropanol ATPS, and $Na_3C_6H_5O_7$/isopropanol ATPS. The phosphate buffer solutions (pH = 3.0–8.0) were prepared using Na_2HPO_4 and NaH_2PO_4. The phase-forming salt solutions were prepared using buffer solutions of different pH values, and then corresponding amounts of alcohol were added to form ATPS. Duplicate analysis as performed for each sample, and the average value was taken to investigate the effect of pH on the partition behavior of flavonoids in the aqueous two-phase systems.

The extraction yield of flavonoids (Y %) was defined as the percentage of flavonoids distributed in the top phase (g_{top}) and the total mixture (g_{total}), and calculated using the following formula:

$$Y\% = \frac{g_{top}}{g_{total}} \times 100\% \quad (1)$$

2.3.3. Aqueous two-phase systems experiment of honey sample

The $Na_3C_6H_5O_7$/isopropanol ATPS was constructed by weighing the predetermined amount of water, isopropanol, $Na_3C_6H_5O_7$ (pH 6.0), isopropanol standard solutions or 1.0 g of honey. The mixture was placed in the ultrasonic bath, extracted for 20 min at 40 ℃, allowed to stand for another 30.0 min at 40 ℃, and then centrifuged at 10,000× g

for 10.0 min using an H2050R centrifuge (Hunan XiangYi Laboratory Instrument Development Co., Ltd., China) to achieve proper phase separation. The volume of the top and bottom phases was estimated visually in the graduated tubes. Then, 2.0 mL of the top phase was taken out carefully with the help of a micropipette to determine the concentration of flavonoids. The final extraction solution was blown to 1.0 mL with nitrogen at 50 ℃, the diluted to 5.0 mL with the mobile phase solution, and finally, passed through a 0.22 μm filter membrane for HPLC-DAD experiments. Duplicate analysis as performed for each sample, and HPLC-DAD was measured n random order according to the sample number.

2.4. Solid phase extraction experiments of honey samples

In this study, the effect of three solid phase extraction columns (HLB, PSD, and MAX) on flavonoids extraction was investigated. The SPE experiment included the following six steps. (1) Sample pretreatment: 1.0 g of honey was dissolved in 3.0 mL of acidified water (HLB and PSD, 0.1% formic acid) or ultrapure water (MAX), vortexed evenly, and then sonicated for 20.0 min at no higher than 50 ℃ until completely mixed; (2) SPE activation: 3.0 mL of methanol was added to activate the SPE column, and then 3.0 mL of acidified water (HLB and PSD, 0.1% formic acid) or ultrapure water (MAX) was added to balance the column; (3) Sample loading: the well-mixed honey samples were added to three SPE columns, respectively; (4) Rinse: the SPE column was rinsed with 3.0 mL of acidified water (HLB and PSD, 0.1% formic acid) or ultrapure water (MAX) to remove sugar and other impurities from honey, and then blown dry with ear ball; (5) Elution: 5.0 mL of methanol was added to elute the SPE column and enrich the flavonoids of honey, and then blown dry with ear ball; (6) Nitrogen blowing: the eluent was blown up to 0.5 mL with nitrogen at a temperature below 50 ℃, then diluted to 2.0 mL with the mobile phase solution, vortexed evenly, and finally, passed through a 0.22 μm filter membrane for HPLC-DAD experiments. Duplicate analysis as performed for each sample, and HPLC-DAD was measured n random order according to the sample number. In addition, the detailed experimental methods for traditional organic phase extraction were described in detail in the **Appendix A**.

2.5. HPLC-DAD conditions

The HPLC-DAD experiment was performed under the following conditions. (1) Instrument: 1260 liquid chromatography system (Agilent Technologies, USA) equipped with a diode array detector (DAD); (2) Column: Agilent Eclipse XDB-C18 column (4.6 mm × 150 mm, 5.0 μm); (3) Mobile phase: using isocratic elution with acidified methanol (0.1% formic acid) and acidified water (0.1% formic acid) at a ratio of 75: 25 and at a flow rate of 1.00 mL/min; (4) Column temperature: 30 ℃; (5) Sample injection volume: 20.0 μL; (6) Wavelength: 190–400 nm with an interval of 2.0 nm. In addition, the detailed conditions for HPLC-DAD method in full-separation mode were referred to **Appendix A**.

2.6. Chemometric analysis

2.6.1. Data analysis

The full spectral data of each sample was obtained by a DAD detector, and a three-way data array was obtained through multiple HPLC scans of multiple samples. Each data point recorded in each chromatographic run was a function of retention time and absorption wavelength. The entire chromatographic data was segmented into four regions according to the retention time of the target analytes to simplify the analysis process and reduce the interference of other irrelevant information. The following compositions were specified: region 1 involved analytes EC and RU, region 2 involved co-elution chromatographic peaks of MY and FI, region 3 involved co-elution chromatographic peaks of QU and HE, and region 4 involved only the analyte KA.

2.6.2. ATLD algorithm

For HPLC analysis, the HPLC data (*.*csv*) was converted into MATLAB m-file (*.*m*) by a self-made program, then imported into MATLAB for further analysis. Then, the outcome of each sample was matrix data with a size of $I \times J$ (retention time × absorption wavelength). A three-way data array ($\underline{\mathbf{X}}$) with a size of $I \times J \times K$ was formed by scanning the K samples. According to the trilinear model, each element (x_{ijk}) in $\underline{\mathbf{X}}$ ($I \times J \times K$) was expressed as follows:

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

where, a_{in}, b_{jn}, and c_{kn} represent the elements of **A** (chromatographic matrix, $I \times N$), **B** (spectral matrix, $J \times N$), and **C** (concentration matrix, $K \times N$), respectively. e_{ijk} represents the element of the three-way residual matrix ($\underline{\mathbf{E}}$) with the same size of $I \times J \times K$. N represents the total number of factors, including the analytes, unknown interferents, baseline shift, instrument noise, and even slight retention time shift. Later, the alternating trilinear decomposition (ATLD) algorithm was applied to correlate the concentration of the analytes with its HPLC-DAD signal. The ATLD algorithm is a well-known iterative algorithm to decompose the three-way data array by alternatively minimizing three objective functions to update **A**, B, and **C**, which has the advantages of being insensitive to the number of factors and fast convergence (Wu, Shibukawa, & Oguma, 1998).

All calculations were performed on MATLAB software and run under the 3.50 GHz Intel (R) Core (TM) *i*5-4690 CPU, 8.00 GB RAM, and Windows 7 operating system. All the codes of the ATLD algorithm can be found in chapter 3 of "Fundamentals and Analytical Applications of Multiway Calibration" monograph (Olivieri, et al., 2015).

3. Results and discussion

3.1. Partition behaviors of flavonoids in ATPS

Compared with other ionic liquid/salt, PEG/salt, surfactant and polymer ATPS, this work selected the simplest and most effective alcohol/salt ATPS to separate impurities and improve the extraction rate of flavonoids in honey. According to the previous studies (Enriquez-Ochoa, et al., 2020; Zhang, et al., 2022; Zhang, et al., 2023), the short-chain alcohols, such as ethanol, *n*-propanol, and isopropanol were selected due to their wide range and low viscosity to construct ATPS for extracting flavonoids from honey. Compared to other types of phase-forming salts, $Na_3C_6H_5O_7$ (or $(NH_4)_2SO_4$) has the advantages of easy availability of raw materials, low price, environmental friendliness, low viscosity of medium and convenient industrialized large-scale production, etc. It has good solubility and phase-forming ability, which has been widely used as a phase-forming salt for the extraction of active compounds in complex systems. The turbidity titration method was used to draw the phase diagrams of $(NH_4)_2SO_4$/alcohol ATPS and the $Na_3C_6H_5O_7$/alcohol ATPS, so as to select the appropriate ratio of phase composition. When adding inorganic salt solution ($Na_3C_6H_5O_7$ or $(NH_4)_2SO_4$) to small molecular alcohol (ethanol, *n*-propanol and isopropanol), and using the salting-out effect to make the salt ions capture the water molecules hydrated with the alcohol to release the alcohol molecules, the alcohol/salt ATPS could be formed. As depicted in Fig. 1, the ability of alcohols to form ATPS was in the following order: *n*-propanol > isopropanol > ethanol, while for salts, the order was $(NH_4)_2SO_4$ > $Na_3C_6H_5O_7$. The phase-separation ability of the studied systems was related to the carbon chain length and molecular polarity of the phase-forming alcohol. The alcohol with a longer carbon chain and less polarity is more hydrophobic and more prone to form an ATPS. Thus, the ability to form ATPS was *n*-propanol > isopropanol > ethanol. Furthermore, due to the large negative Gibbs free energy value

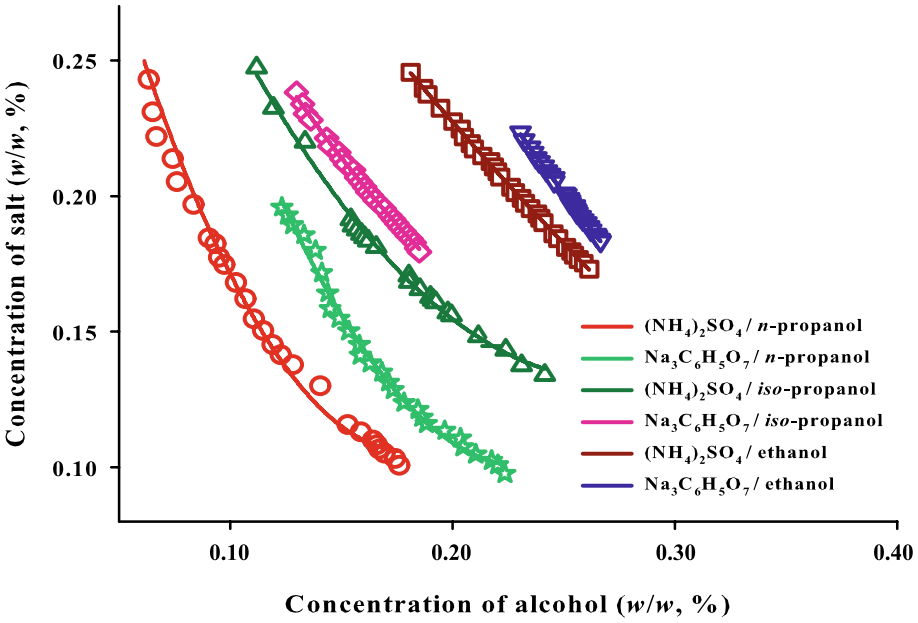


Fig. 1. The bimodal curves of the alcohol-salt ATPS.

of hydration, the salting-out ability of $(NH_4)_2SO_4$ was greater than that of $Na_3C_6H_5O_7$, so the ability to form ATPS was $(NH_4)_2SO_4$ > $Na_3C_6H_5O_7$. In Fig. 1, each curve represents two zones, and the upper part of the curve was the two-phase zone. In this zone, the top phase was the alcohol-rich phase, and the bottom phase was the salt-rich phase. Therefore, in the following experiments, the concentration of alcohol and salt was selected in the zone above the curve.

Six types of ATPS composed of alcohols and salts (e.g. $(NH_4)_2SO_4$/ethanol, $Na_3C_6H_5O_7$/ethanol, $(NH_4)_2SO_4$/*n*-propanol, $Na_3C_6H_5O_7$/*n*-propanol, $(NH_4)_2SO_4$/isopropanol, and $Na_3C_6H_5O_7$/isopropanol) were studied to verify the partition behavior of flavonoids in ATPS. The composition of the six types of ATPS remained the same. The ratio between 30% salt, water, and alcohol was 2.82 (mL): 1.58 (mL): 3.10 (mL). Flavonoids are unstable active components. Hence, too high temperature and too long ultrasound make them prone to be damaged by the chemical reactions, such as oxidation and polymerization, decreasing the extraction rate. Referring to the literature (Ran, et al., 2019; Zhang, et al., 2023), extraction temperature and ultrasonic time were set as 40 ℃ and 20.0 min, respectively. The top phase recovery yield of flavonoids in the alcohol-salt ATPS was depicted in Fig. 2. It could be seen from Fig. 2 that the extraction efficiency of the ATPS constructed with isopropanol as the alcohol phase was better than ethanol and *n*-propanol. As for the $(NH_4)_2SO_4$/isopropanol ATPS, the top phase recovery yield of flavonoids was greater than or equal to 56.72% under pH = 3.0–6.0. While, for the $Na_3C_6H_5O_7$/isopropanol ATPS, the top phase recovery yield of flavonoids was greater than or equal to 79.25% under the same pH values. These results suggested that the $Na_3C_6H_5O_7$/isopropanol ATPS was more suitable for the extraction of flavonoids from honey. Especially for EC, the top phase extraction rates of $(NH_4)_2SO_4$/isopropanol ATPS were generally low, only 70.16–76.03%, while the top phase extraction fields of $Na_3C_6H_5O_7$/isopropanol ATPS could reach 81.13–101.26%. The main reason why $Na_3C_6H_5O_7$/isopropanol ATPS exhibited better extraction of flavonoids than $(NH_4)_2SO_4$/isopropanol might be the interaction of the analytes with the ATPS components. The important flavonoids identified in honey, all characterized by the presence of an *x*-phenyl-1,4-benzopyrone backbone (where $x = 2, 3$), which contain a large number of functional groups, such as –OH, $–COO^-$, etc. These functional groups might interact with NH_4^+, making it easier to distribute in the bottom phase of ATPS, resulting in lower extraction efficiency.

To investigate the influence of pH, the extraction flavonoids from honey samples using $Na_3C_6H_5O_7$/isopropanol ATPS were performed under different pH value as illustrated in Fig. 2. As for seven flavonoids (especially MY and QU), the extraction efficiency of an acidic environment was better than the neutral and alkaline conditions. The extraction yields were satisfactory within the range of 87.66–101.50% under pH = 6.0. The extraction rate of flavonoids showed a downward trend with the increase of pH value. The reason for this phenomenon might be attributed to the following aspects (Wang, et al., 2018; Wang et al., 2021a): (1) The degree of protonation of the citrate ions varied at different pH values, which would change the extent of interactions (analytes–ATPS components) in the ATPS. When the pH value is greater than 6.0, the polarity of flavonoids increases with the increase of pH, the ability to capture for water molecules is enhanced, and it is easier to enter the hydrophilic lower phase, resulting in a decrease in the extraction rate; (2) The electrostatic interaction between the two phases was influenced by the pH value of the solutions, which resulted in the change in extraction yield values of the flavonoids; (3) The flavonoids were prone to form salts and assumed an anionic state under neutral and alkaline conditions. This state promoted the formation of strong hydrogen bonds with the water molecules, making it easier to distribute in the bottom phase of ATPS, which caused the extraction yield decrease; (4) Flavonoids were prone to oxidative polymerization at high pH values, thereby decreasing the extraction rate.

According to the above experimental results, it was found that the $Na_3C_6H_5O_7$/isopropanol ATPS was more suitable than other alcohols/salt ATPS for the extraction of flavonoids in honey. The extraction yields were satisfactory under acidic environment (pH = 6.0). Moreover, the extraction of active substances from honey using ATPS has some advantages, such as mild conditions, cost-effectiveness, and reduced organic solvents, suggesting this technology to be beneficial for industrial amplification and extraction of easily oxidized active substances under high temperatures. Therefore, the pH 6.0 $Na_3C_6H_5O_7$/isopropanol ATPS was selected to combine with the chemometrics-assisted HPLC-DAD for the simultaneous extraction and determination of flavonoids in honey.

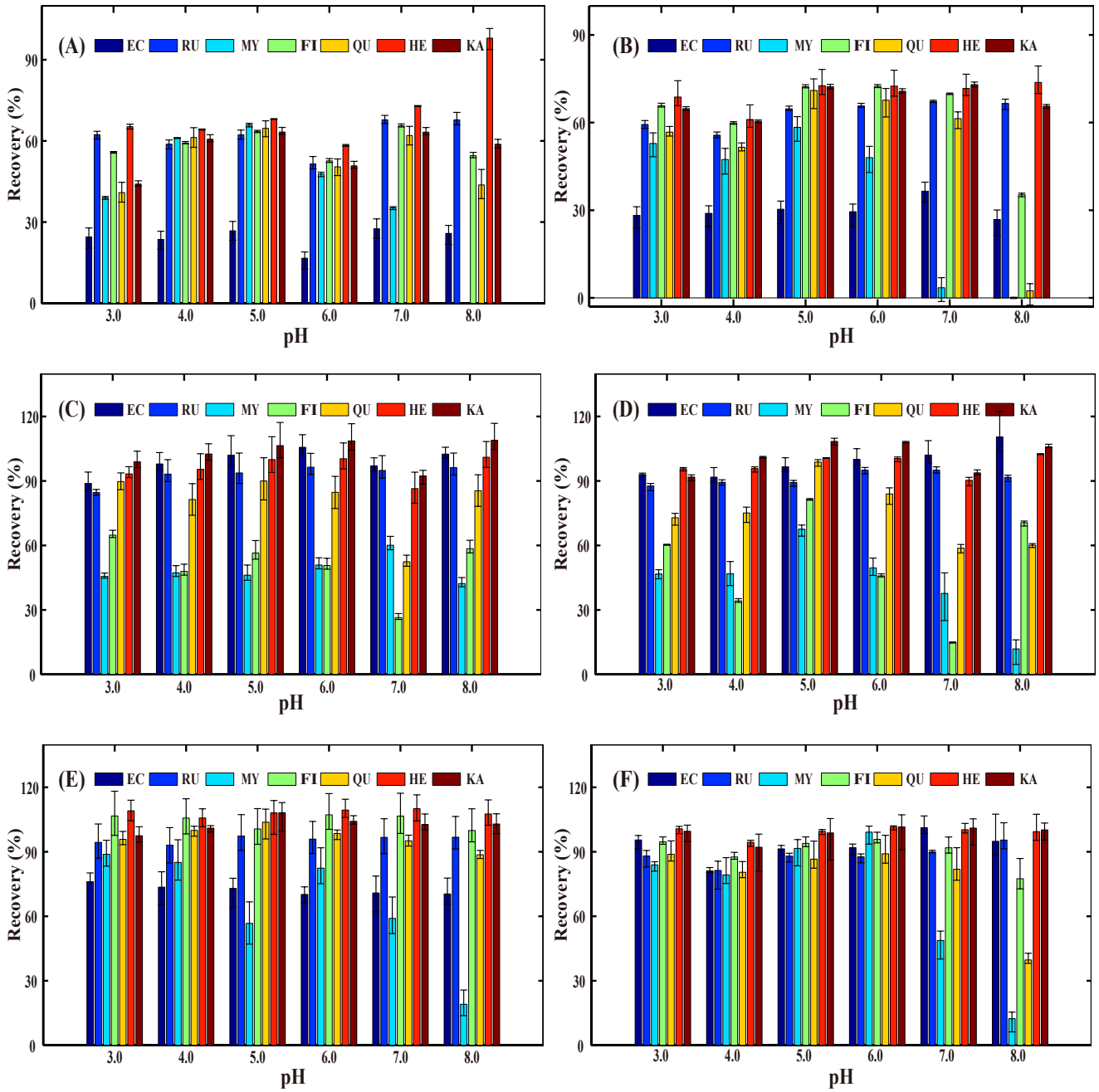


Fig. 2. The top phase recovery yield of flavonoids in different alcohol-salt ATPS. (A) $(NH_4)_2SO_4$/ethanol ATPS; (B) $Na_3C_6H_5O_7$/ethanol ATPS; (C) $(NH_4)_2SO_4$/*n*-propanol ATPS; (D) $Na_3C_6H_5O_7$/*n*-propanol ATPS; (E) $(NH_4)_2SO_4$/isopropanol ATPS; (F) $Na_3C_6H_5O_7$/isopropanol ATPS.

3.2. Chemometric analysis

3.2.1. Three-way data preprocessing

The three-way array (retention time × absorption wavelength × sample) was obtained through multiple HPLC scans of multiple honey samples. These arrays contained many useful chemical informations, response of unknown interferents, background and/or instrument noise (Fig. 3C). Therefore, it was impossible to perform an accurate quantitative analysis of flavonoids in honey using the traditional univariate calibration methods. As depicted in Fig. 3A and B, the seven flavonoids were completely eluted within 4.5 min, but there was a co-elution phenomenon in the retention time dimension, such as the high peak overlapping between EC and RU, MY and FI. This phenomenon was more severe in real honey samples due to the complex background matrix and various unknown interferents. In such a case, the overlapping spectra can be divided into the spectra of each pure analyte and interferents by the mathematical methods. This is the so-called "second-order advantage", i.e., the qualitative and quantitative analysis of the target analytes can be quickly and accurately achieved under the condition of overlapping peaks and unknown interferents coexistence.

Besides, the retention time shift is another common phenomenon in the chromatographic analysis, affecting the accuracy of the resolution results of the second-order calibration algorithm. The typical chromatograms of eight calibration samples at the wavelength of 274 nm had been superimposed in Fig. 3B to judge the trilinear property of the data. Due to the isocratic elution mode, baseline drift and slight retention time shift could be observed. However, the ATLD algorithm with the mathematical strategies could overcome the above-mentioned problems and obtain accurate quantitative analysis results (Olivieri & Escandar, 2019;

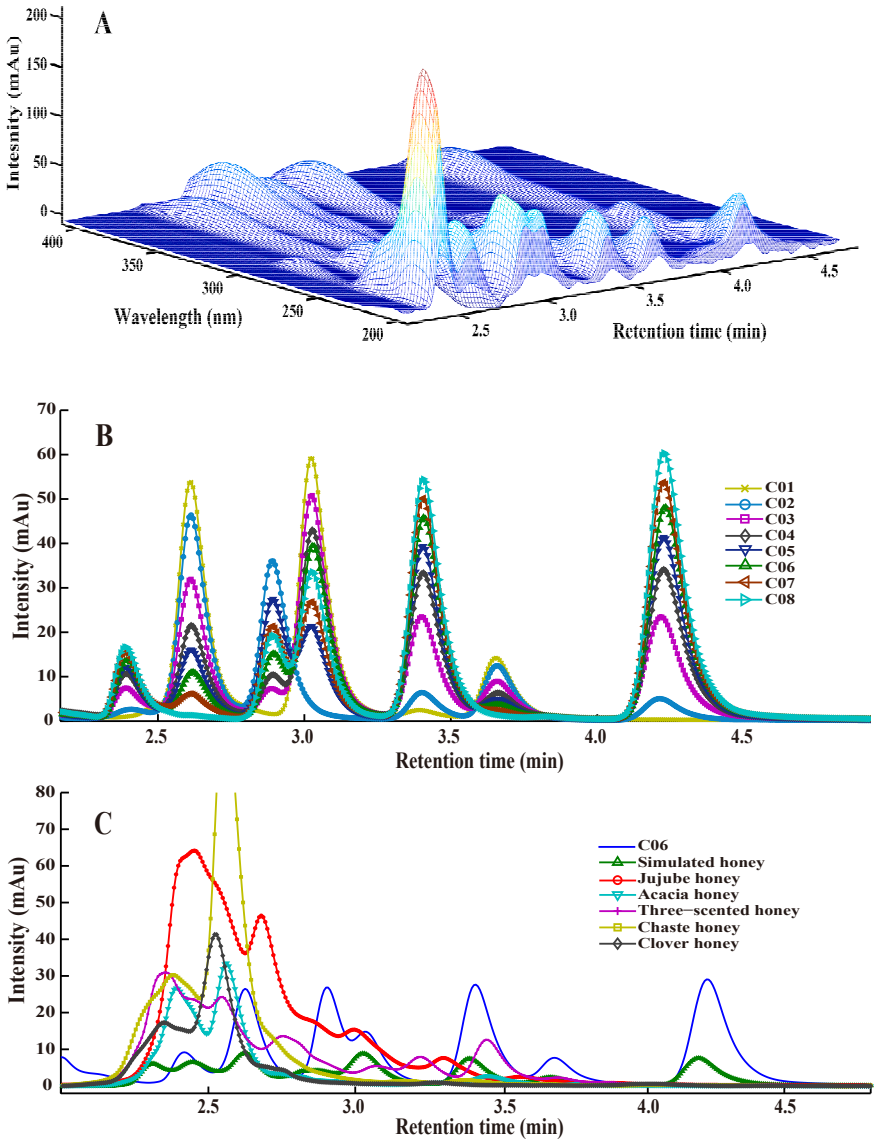


Fig. 3. (A) Three-way chromatographic-spectral landscape of calibration sample C06; (B) typical stacked chromatograms (λ = 274 nm) of eight calibration samples; (C) two-dimensional stacked chromatograms (λ = 274 nm) of calibration sample C06, simulated honey sample and five types of honey samples from different floral origins.

Zhang, Qing, & Wu, 2019). During the experiment, if there are obvious retention time shifts, appropriate time alignment programs can be added in the data preprocessing process. In addition, other multivariate calibration algorithms overcoming the retention time drift can be chosen, such as multivariate curve resolution-alternating least squares (MCR-ALS), parallel factor analysis 2 (PARAFAC2), alternating trilinear decomposition-assisted multivariate curve resolution (ATLD-MCR), etc. (Bro, Andersson, & Kiers, 1999; Kiers, Berge, & Bro, 1999; Olivieri, et al., 2015; Tauler, 1995; Zhang, et al., 2020).

3.2.2. *ATLD modelling*

In this study, eight calibration samples were used to establish the ATLD algorithm model, and a pseudo-univariate calibration curve was constructed by regressing the actual concentration (x) of each target analyte in the calibration samples to its ATLD resolved score (y). The corresponding regression equations and the correlation coefficients (R^2) of the seven flavonoids were depicted in Fig. S1. The linear regression equations of all target analytes had good correlation coefficients (R^2 >0.9948), indicating good linear relations among the actual concentrations and ATLD resolved scores of seven flavonoids in the calibration samples. This experiment also designed three validation samples to verify the accuracy and resolution efficiency of the developed ATLD algorithm model. The quantitative prediction results of the analytes corresponding to the validation set were summarized in Table 1. The average recovery rates of the seven target analytes were 93.21–100.28%, and the standard deviations were less than 4.51%, indicating the accuracy and reliability of the established ATLD algorithm model.

An obvious conclusion can be drawn from the above discussion that the established ATLD model is a good choice for processing second-order chromatographic data with baseline drift and slight retention time shift to obtain the quantitative result of analytes in complex systems with

Table 1
ATLD prediction results of the analytes corresponding to the validation set.

Analytes	Real concentrations ($\times10^3$ ng/mL)			Prediction concentrations ($\times10^3$ ng/mL)			R^2 [b]	AVG ± SD (%) [c]
	V01	V02	V03	V01	V02	V03		
EC [a]	2.01	3.02	5.03	1.95	3.00	4.97	0.9998	98.53±1.02
RU	9.65	8.44	6.03	9.95	8.13	5.63	0.9956	97.59±3.73
MY	0.77	6.95	6.18	0.72	7.26	6.35	0.9980	100.28±4.51
FI	16.44	1.83	3.65	16.43	1.72	3.51	0.9996	96.64±2.18
QU	2.01	3.02	5.03	1.98	2.87	4.84	0.9956	96.68±1.12
HE	3.17	2.77	1.98	3.15	2.79	1.96	0.9996	99.70±0.54
KA	2.09	3.14	5.23	2.00	2.86	4.83	0.9948	93.21±1.77

[a] EC: epicatechin; RU: rutin; MY: myricetin; FI: fisetin; QU: quercetin; HE: hesperidin; KA: kaempferol.
[b] R^2 is the square of correlation coefficient.
[c] AVG ± S.D. denotes the average recovery ± standard deviation.

uncalibrated interferents. Accordingly, the efficacy of the ATLD-based second-order calibration method for rapid quantitative analysis of flavonoids in honey was further evaluated.

3.3. Targeted quantitative analysis of flavonoids using ATPS-ATLD-based method

3.3.1. Quantification in simulated honey samples

Honey, a supersaturated solution of sugar, possesses a complex background matrix. Therefore, before using the established ATPS-ATLD-based method to extract and quantify flavonoids in honey, a series of simulated honey samples were configured to verify its reliability and accuracy. The simulated honey was prepared to simulate the composition of honey by adding the proper volume of stock solutions of seven flavonoids to corn syrup. The detailed preparation method was described in section 2.2 of the paper. The ATLD algorithm was used to decompose the three-way data array of each region of the simulated honey samples. The results showed that although there were unknown interferents and strong background in the simulated honey, accurate and reasonable chromatograms, spectra and concentration profiles of each analyte could also be resolved (Fig. 4). When the ratio of the simulated honey mass to the volume of the $Na_3C_6H_5O_7$/isopropanol ATPS was 0.38 g: 7.5 mL, the extraction yields of the seven flavonoids were EC 91.27%, RU 91.77%, MY 92.35%, FI 99.04%, QU 102.08%, HE 88.68%, and KA 111.03%, respectively. Similarly, when the ratio of the simulated honey mass to the volume of the $Na_3C_6H_5O_7$/isopropanol ATPS was 0.51 g: 7.5 mL, the extraction yields of the seven flavonoids were EC 96.99%, RU 93.55%, MY 99.71%, FI 106.92%, QU 113.29%, HE 89.09%, and KA 114.10%, respectively. The above results showed that the established ATPS-ATLD-based method was accurate and reliable, and could be used for the extraction and quantitative analysis of flavonoids in honey.

3.3.2. Quantification in real honey samples

In this study, seven analytes (including EC, RU, MY, FI, QU, HE, and KA) in five types of honey samples from different floral origins (including jujube, acacia, clover, three-scented, and chaste) were analyzed. After the ATLD algorithm was used to decompose the three-way data array of five real honey samples, the chromatograms (**A**), spectra (**B**), and concentration profiles (**C**) were obtained. The resolved profiles of chaste honey samples were depicted in Fig. 5, while for other types of honey, the obtained results remained the same. It could be seen that the chromatogram and spectra of the target analytes overlapped heavily with the unknown interferents in honey. For instance, three unknown interferents overlapped with the profiles of EC and RU in region 1, one unknown interferent overlapped with the profiles of MY and FI in region 2, and one unknown interferent overlapped with the profiles of HE and QU in region 3. Additionally, the contents of the unknown interferents in the chaste honey were relatively high, which was comparable to the analytes (Fig. 5**C1)**. Despite these problems, the established ATPS-ATLD-based method could still obtain accurate quantitative analysis results, as shown in Table 2. The contents of flavonoids in the five different types of honey measured by the HPLC-DAD were in quite different ranges, and the average spiked recoveries were between an acceptable range of 84.12–115.10%.

The quantitative results obtained by established ATPS-ATLD-based method were satisfactory considering the complexity of the real honey samples, simple sample pretreatment, simple elution mode, complicated matrix interferences and highly overlapping peaks. It was demonstrated that this analytical strategy was extremely promising for the identification and quantification of analytes in the complex samples. As summarized in Table 2, there were significant differences in the contents and types of flavonoids in honey obtained from different floral origins. This might be attributed to the flower source, some environmental factors (such as season, temperature, climate, soil, etc.), and might be due to the quality of honey.

3.4. Targeted quantitative analysis of flavonoids using SPE-ATLD-based method

The SPE method is a “golden standard” method for extracting the active components from complex matrices. Herein, the analysis results of this “golden standard” method were used as a control experiment to verify the reliability of the developed ATPS-ATLD-based method. Firstly, three SPE columns (HLB, PSD and MAX) were selected to extract the flavonoids from honey based on the physical and chemical properties of the target analytes. According to the ATLD prediction results (Table S2), the extraction effect of HLB was poor, and the extraction yields of RU reached 85.80%, while other analytes were less than 70%, and QU did not achieve the phenomenon of effective adsorption. Fortunately, the extraction effects of PSD and MAX were meeting the related requirements, and the extraction yield of the analyte reached more than 75%. Of which, the extraction yields of PSD reached were the best, within the range of 79.03–97.90%. Therefore, for subsequent SPE experiments, the extraction of flavonoids from honey was performed on the PSD solid-phase extraction columns.

The contents (ng/g) and average extraction yields (%) of seven flavonoids in the five types of honey samples from different floral origins predicted by the Poly-Sery PSD SPE-ATLD-based method were listed in Table 3. It could be seen that most of the analytes showed good spiked recovery rates, but the recovery rates of EC in five types of honey were low (48.06–67.96%), and the recovery rates of MY in the three fragrant nectar and chaste honey were high (132.62% and 121.81%, respectively). Similarly, the recovery rates of QU and KA in the acacia honey were also high (125.25% and 124.68%, respectively), which could not meet the experimental requirements.

The above experimental results showed that the separation and purification of flavonoids in honey by SPE was not satisfactory. This might be attributed to the physical and chemical properties of EC, the composition of the PSD solid phase extraction column and the principle of adsorption and desorption.

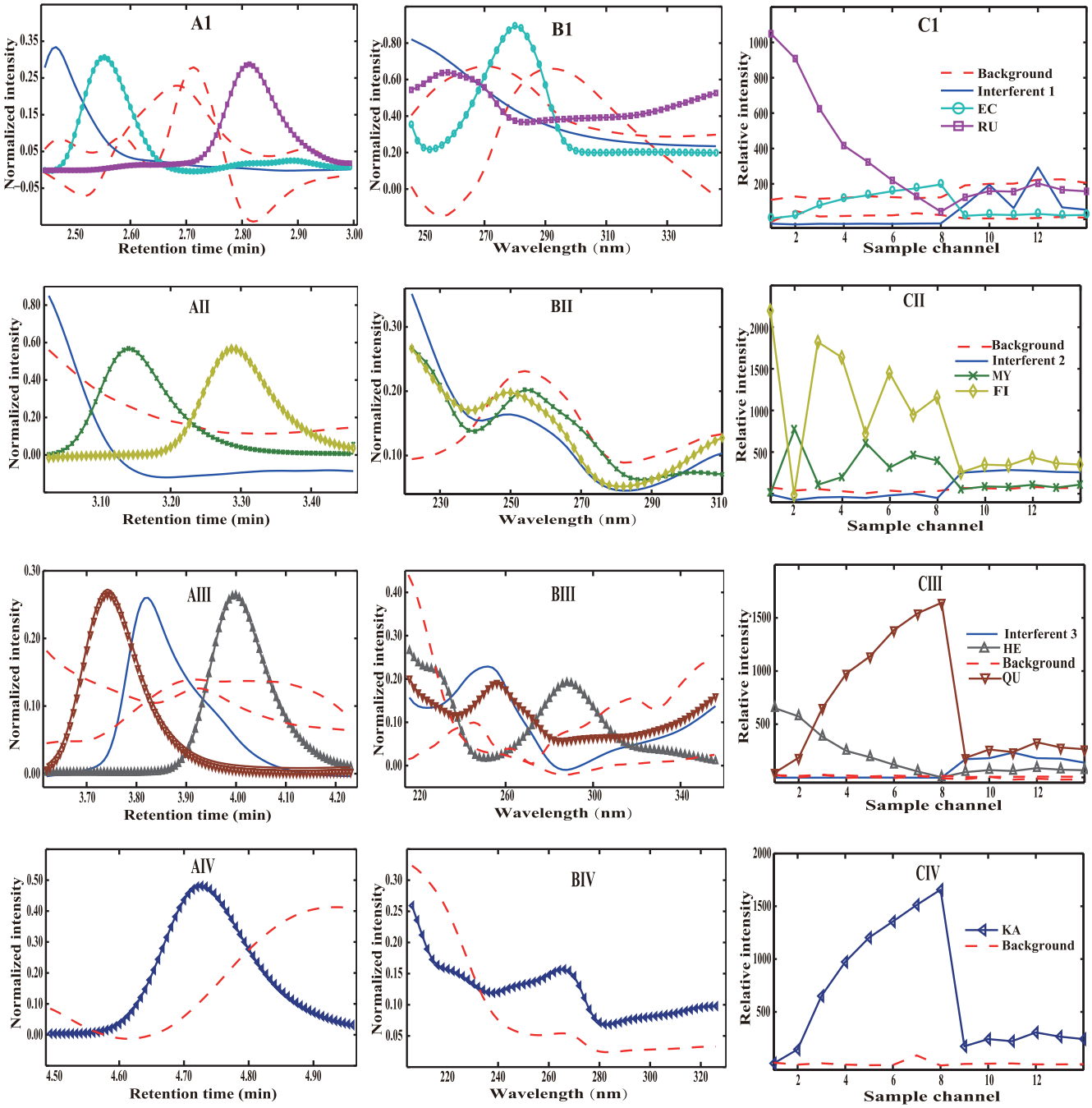


Fig. 4. The chromatograms (AI-AIV), spectra (BI-BIV) and concentration profiles (CI-CIV) resolved by the ATLD algorithm for simulated honey samples. The marker lines are flavonoids, the blue and red lines are interferents and background, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Method performance comparison

The quantitative results of the proposed method and the SPE method were statistically compared and listed in Table 4. It could be seen that except for EC, the contents of other six analytes in the clover honey predicted by the two methods were similar. The predicted content of EC in the clover honey by SPE was only half of the content obtained by the ATPS extraction method, and the spiked recovery was also about half, suggesting that the established ATPS-ATLD-based method has more advantages and could be applied for the extraction and quantification of flavonoids in honey. Additionally, the validation parameters were calculated, including the limit of detection (LOD), the limit of quantification (LOQ), the sensitivity (SEN), the selectivity (SEL), and the average recovery (AVG) to compare the performance of the ATPS-ATLD-based method and SPE-ATLD-based method. Considering the complexity of the honey matrix and the presence of a high concentration of interferents in the retention time region of the target analytes, the results of these two methods were found satisfactory. However, compared with the SPE-ATLD-based method, the ATPS-ATLD-based method had lower LOD (36.90–46.38 ng/g), and higher SEN (1.31×10^3–8.37×10^3 g/ng) (Table 4). These results further verified the accuracy and stability of the established method.

Moreover, as a comparison, the qualitative and quantitative analysis of target analytes was performed based on traditional organic phase

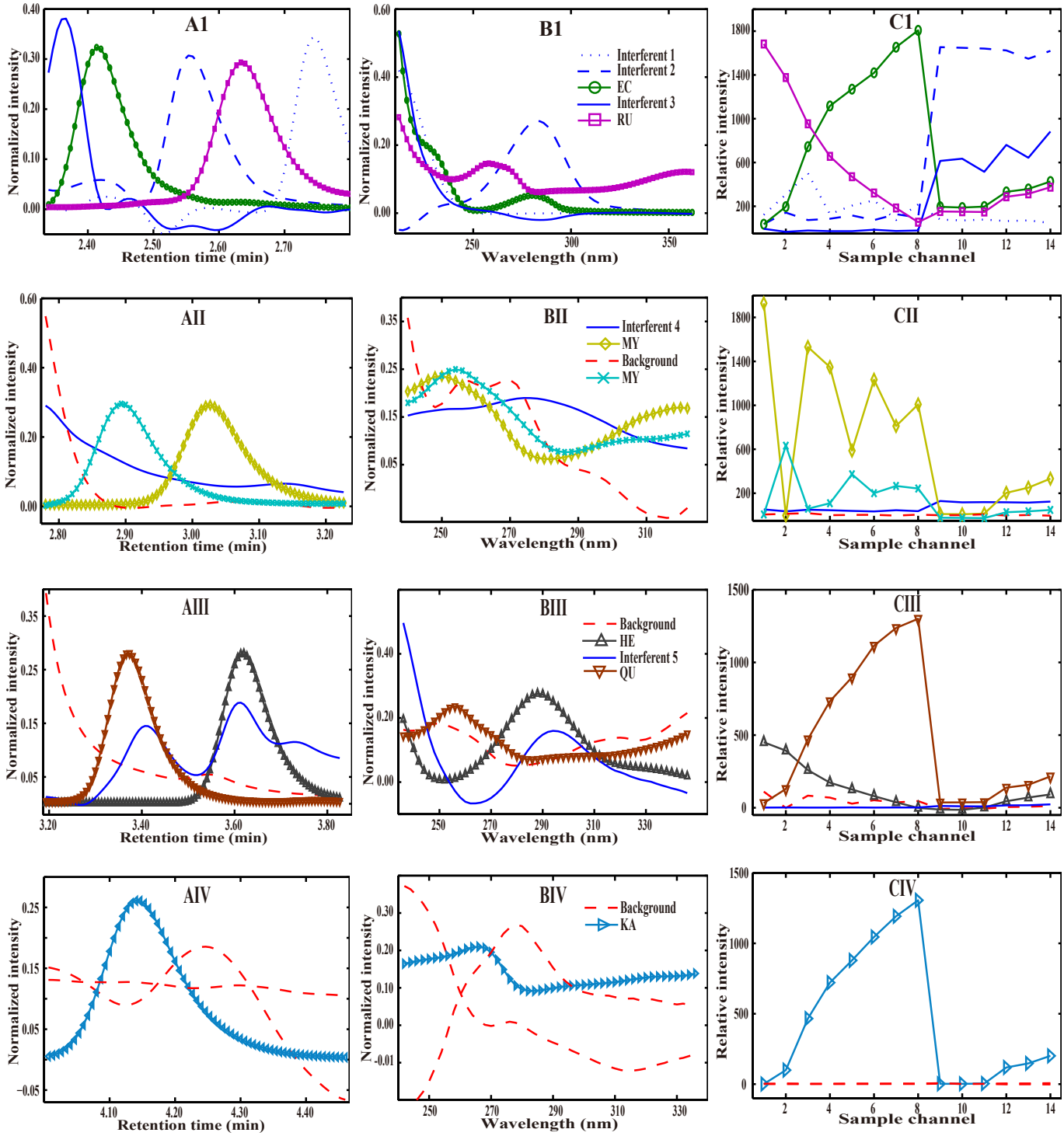


Fig. 5. The chromatograms (AI-AIV), spectra (BI-BIV) and concentration profiles (CI-CIV) resolved by the ATLD algorithm for chaste honey samples. The marker lines are flavonoids, the blue and red lines are interferents and background, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

extraction combined with HPLC-DAD method in full-separation mode, and the obtained results were shown in Table S3 and Fig. S2 in the **Appendix A**. As depicted in Fig. S2, the traditional full-separation HPLC-DAD took 16.0 min for each honey sample, while the ATPS-ATLD-based method only needed 4.5 min. Thus, the method constructed in this paper could save more experiment time and reduce the use of organic reagents. As shown in Table S3, with the exception of HE and FI, the results obtained using the ATPS-ATLD-based method are similar and in the same order of magnitude as those obtained in the above traditional method. The most important reasons for the difference in the quantitative results of HE and FI may be that the contents in honey are relatively low, and the extraction methods are quite different. However, the pretreatment methods and chromatographic conditions of conventional methods are too complicated (see **Appendix A**), and often occupies most of the time of the entire experiment, greatly reducing the efficiency of the experiment. Moreover, traditional methods use a large

Table 2
Contents (ng/g) and average recoveries (%) of flavonoids in honeys predicted by the ATPS-ATLD-based method.

Analytes	Honey 1[b]	Honey 2	Honey 3	Honey 4	Honey 5
EC [a]	2470.97 (90.08 ±3.97) [c]	7334.60 (102.07 ±2.78)	6545.63 (106.41 ±2.77)	9762.90 (95.51 ±5.33)	7693.31 (97.64 ±5.12)
RU	9620.97 (96.12 ±6.48)	5223.05 (88.01 ±2.53)	13441.50 (115.10 ±4.34)	10659.95 (95.12 ±5.28)	3454.25 (87.90 ±3.18)
MY	4390.10 (113.71 ±1.92)	5463.85 (104.43 ±5.02)	1734.38 (95.40 ±7.86)	5173.70 (89.27 ±7.19)	5952.86 (110.23 ±5.57)
FI	- [d](84.12 ±2.17)	-(88.72 ±1.09)	-(90.10 ±1.35)	-(92.00 ±2.75)	-(101.28 ±1.62)
QU	-(108.13 ±1.73)	4490.15 (100.26 ±2.07)	-(88.37 ±0.38)	4102.70 (94.40 ±4.45)	1632.14 (93.04 ±0.70)
HE	270.97 (89.98 ±3.74)	254.63 (88.12 ±1.32)	2011.13 (113.86 ±2.66)	-(98.01 ±5.10)	419.23 (101.88 ±3.20)
KA	2442.00 (86.29 ±1.20)	7105.70 (96.16 ±3.59)	2023.13 (104.59 ±1.19)	3298.75 (84.54 ±2.57)	2468.88 (86.15 ±2.41)

[a] EC: epicatechin; RU: rutin; MY: myricetin; FI: fisetin; QU: quercetin; HE: hesperidin; KA: kaempferol.
[b] Honeys: Honey 1, jujube flower honey; Honey 2, acacia honey; Honey 3, **three fragrant nectar**; Honey 4, chaste honey; Honey 5, clover honey.
[c] Average recovery ± standard deviation (%) in parenthesis.
[d] - denotes not detected.

Table 3
Contents (ng/g) and average recoveries (%) of flavonoids in honeys predicted by the Poly-Sery PSD SPE-ATLD-based method.

Analytes	Honey 1[b]	Honey 2	Honey 3	Honey 4	Honey 5
EC [a]	1765.83 (52.73 ±12.40) [c]	8541.33 (48.06 ±12.49)	6304.00 (67.96 ±15.34)	9700.00 (54.61 ±9.43)	3982.50 (54.27 ±18.03)
RU	9290.50 (93.17 ±3.57)	5553.50 (80.02 ±3.74)	13018.83 (75.31 ±1.22)	10860.67 (82.01 ±2.43)	3839.00 (98.11 ±1.43)
MY	4401.17 (111.65 ±2.19)	5826.33 (116.16 ±13.89)	1790.67 (132.62 ±4.03)	5234.67 (121.81 ±4.39)	5623.83 (81.00 ±4.80)
FI	- [d](103.51 ±3.58)	-(98.50 ±9.91)	-(107.30 ±6.02)	-(100.50 ±1.74)	-(95.04 ±3.28)
QU	-(95.09 ±5.55)	4854.50 (125.25 ±3.73)	-(97.27 ±5.82)	4446.17 (109.09 ±1.36)	1793.33 (97.86 ±2.44)
HE	280.33 (91.07 ±0.46)	23.83 (95.07 ±0.49)	2270.83 (98.42 ±1.30)	-(98.47 ±0.91)	315.83 (106.01 ±0.70)
KA	2147.17 (104.96 ±0.83)	7172.00 (124.68 ±1.35)	1652.00 (81.46 ±2.82)	3119.33 (76.05 ±0.14)	2630.50 (95.63 ±1.42)

[a] EC: epicatechin; RU: rutin; MY: myricetin; FI: fisetin; QU: quercetin; HE: hesperidin; KA: kaempferol.
[b] Honeys: Honey 1, jujube flower honey; Honey 2, acacia honey; **Honey 3, three fragrant nectar;** Honey 4, chaste honey; Honey 5, clover honey.
[c] Average recovery ± standard deviation (%) in parenthesis.
[d] - denotes not detected.

number of organic solvents, which have adverse effects on the environment and human health. Relatively speaking, the ATPS-ATLD-based method constructed in this work has many advantages, such as: saving solvent and operating time, and thereby reducing the cost per analysis and environmental impact.

Compared with the SPE-based and traditional organic phase extraction methods, the established ATPS-ATLD-based method is reliable, accurate, green, and environmentally friendly, will be extremely promising for the identification and quantification of analytes studied in the complex honey samples. In addition, the quantitative results obtained in this paper are similar to those reported in the literatures (Teodora & Elisabeta-lrina, 2019; Lo Dico, et al., 2019), and are in the same order of magnitude, but the pretreatment method is simpler and effective. For example, compared with the study by Ciucure et al., the ATPS-ATLD-based method used in this work has a simpler pretreatment method and shorter analysis time (Teodora & Elisabeta-lrina, 2019). The mean contents of flavonoids obtained in this work are higher than that reported by Lo Dico, et al. (Lo Dico et al., 2019). The most important reason is that different methods of sample preparation are used, and the method in this work is more effective in removing interferents in honey, so as to effectively extract flavonoids. Besides, the contents and types of flavonoids in different honey samples were quite different, attributing to the factors, such as variety, origin, and climate. Overall, the above discussions and conclusions guide for a reasonable selection and use of ATPS coupled with chemometrics-enhanced HPLC-DAD methods for simultaneous extraction and determination of flavonoids in honey.

4. Conclusions

In this study, a novel extraction and quantitative analysis method was developed, which could efficiently extract and simultaneously determine the flavonoids in five types of honey samples from different floral origins using the aqueous two-phase system coupled with chemometric tools. Firstly, based on the characteristics of active components and impurities, the $Na_3C_6H_5O_7$/isopropanol ATPS was constructed to extract the flavonoids from honey. The extraction process was optimized, and the extraction efficiency was satisfactory within the range of 87.66–101.50% for seven target analytes. Additionally, the three-way array (retention time × absorption wavelength × sample) based on HPLC-DAD was obtained, and the ATLD algorithm was applied for a mathematical resolution to obtain reasonable chromatograms, spectra, and concentration profiles of each analyte under the condition of overlapping peaks and unknown interferents coexistence. Finally, the established ATPS-ATLD-based method was used to extract and quantitatively analyze the flavonoids in simulated and real honey samples. The average spiked recoveries were within an acceptable range of 84.12–115.10%. Compared with SPE and traditional organic phase extraction methods, the ATPS-ATLD-based method showed lower LOD and higher SEN, verifying its accuracy and stability. In summary, the study combined the efficient ATPS extraction method with the second-order calibration method of chemometrics for the first time and realized the simultaneous and rapid quantitative analysis of flavonoids in honey. However, there are still some aspects to be improved in this study. It is necessary to further investigate the influence of salt and alcohol concentration, and temperature on the extraction yields of flavonoids to determine the optimal aqueous two-phase extraction system. In addition, the experimental results showed that there were significant differences in the contents and types of flavonoids in honey obtained from different floral origins. Therefore, we will plan to differentiate the honey origins based on the qualitative and quantitative results obtained.

CRediT authorship contribution statement

Xiao-Hua Zhang: Conceptualization, Methodology, Investigation, Visualization, Funding acquisition, Writing – original draft. **Xiang-Dong Qing:** Conceptualization, Methodology, Software, Funding acquisition. **Jing-Jing Zheng:** Formal analysis, Methodology. **Yan Yu:** Formal analysis, Investigation. **Jiaojiao Huang:** Formal analysis, Investigation. **Chao Kang:** Investigation, Writing – review & editing. **Zhi Liu:** Writing – review & editing, Software, Resources, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

Table 4
Comparison of the $Na_3C_6H_5O_7$/isopropanol ATPS preparation method and the Poly-Sery PSD solid phase extraction preparation method for clover honey.

	EC	RU	MY	FI	QU	HE	KA
$Na_3C_6H_5O_7$/isopropanol ATPS							
Contents (ng/g)	7693.31	3454.25	5952.86	-[a]	1632.14	419.23	2468.88
LOD (ng/g)[b]	225.50	466.38	194.75	398.73	36.90	178.35	132.23
LOQ (ng/g)[c]	682.65	1413.48	589.38	1208.48	111.73	539.15	399.75
SEN × 10^3 (g/ng)[d]	2.25	4.80	1.85	3.04	1.31	8.37	2.62
SEL[e]	0.146	0.490	0.0917	0.115	0.0679	0.357	0.212
AVG ± S.D. (%)[f]	97.64±5.12	87.90±3.18	110.23±5.57	101.28±1.62	93.04±0.70	101.88±3.20	86.15±2.41
Solid phase extraction based on Poly-Sery PSD							
Contents (ng/g)	3982.50	3839.00	5799.00	–	1793.33	315.83	2630.50
LOD (ng/g)	1474.98	410.00	1230.00	1054.73	1716.88	723.65	116.85
LOQ (ng/g)	4467.98	1243.33	3725.88	3194.93	5203.93	2191.45	353.63
SEN × 10^3 (g/ng)	0.91	2.23	1.48	1.40	2.31	4.86	7.26
SEL	0.0742	0.191	0.0815	0.0746	0.147	0.417	0.349
AVG ± S.D. (%)	54.27±18.03	98.11±1.43	81.89±4.55	91.88±4.79	97.86±2.44	106.01±0.70	95.63±1.42

[a] denotes not detected.
[b] Limit of detection was calculated as LOD = 3.3 s(0), where s(0) is the standard deviation of three blank samples for each analyte.
[c] Limit of quantitation was calculated as LOQ = 10 s(0), where s(0) is the standard deviation of three blank samples for each analyte.
[d] $SEN_n = l_n\{[(\mathbf{A}^T_{cal}(\mathbf{I}\text{-}\mathbf{A}_{unx}\mathbf{A}^+_{unx})\mathbf{A}_{cal})^*(\mathbf{B}^T_{cal}(\mathbf{I}\text{-}\mathbf{B}_{unx}\mathbf{B}^+_{unx})\mathbf{B}_{cal})]^{-1}\}_{nn}{}^{-1/2}$, where **A** and **B** are the obtained normalized matrixes from the decomposition of ATLD algorithm, subscript *n* identifies a particular analyte of interest, l_n is the total response signal for *n*th component at unit concentration.
[e] $SEL_n = SEN_n/l_n$.
[f] AVG ± S.D. denotes the average recovery ± standard deviation.

the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

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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.2023.100766.

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