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ORIGINAL

Low-Temperature Cleanup Followed by Dispersive Solid-Phase Extraction for Determination of Nine Polar Plant Growth Regulators in Herbal Matrices Using Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

Polar plant growth regulators, used alone or doped in fertilizers, are most effective and widely utilized plant growth regulators (PGRs) in agriculture, which play important roles in mediating the yield and quality of crops and foodstuffs. The application scope has been extended to herbal medicines in the past 2 decades and relevant study is inadequate. The aim of this study is to establish a QuPPe-based extraction method containing low-temperature and *d*-SPE cleanup procedure followed by the detection on a selective multiresidue ultrahigh-performance liquid chromatography – triple quadrupole tandem mass spectrometry (UHPLC–QqQ–MS/MS) in three herbal matrices. This simple, accurate, versatile and robust method was verified according to the validation criteria of the SANTE/12682/2019 guideline document. The analytical range was from 2.5 to 200 µg/L, and the average recoveries were in the range of 64.6−117.8% ($n=6$). The optimized method was applied to 135 herbal medicines thereof. Result showed that the detection frequency of chlormequat was the highest in the investigated PGRs, with the positive rate of 15.6%. Improvement of the detection method for polar PGRs will enrich the coverage of PGRs, which is conducive to safeguard public health and ensure drug safety.

Keywords Polar plant growth regulators · Herbal medicines · QuPPe · UHPLC– QqQ– MS/MS · Positive rate

Abbreviations

ALAR	Daminozide
AL-N	Alumina N
BNZ	Benzimidazole
C18	Octadecylsilyl
CCC	Chlormequat chloride
CE	Collision energy
CUR	Curtain gas
CXP	Collision cell exit potential
DP	Declustering potential
DA-6	Diethylaminoethylhexanoate
DAD	Diode array detector
DPC	Mepiquat chloride
d-SPE	Dispersive solid-phase extraction
EP	Entrance potential
EURL-SRM	European Reference Laboratory for Single Residue Methods
FA	Fosamine ammonium
FLD	Fluorescence detector
GCB	Graphitized carbon black
HILIC	Hydrophilic interaction liquid chromatography
LOQ	Method quantitation limits
MEs	Matrix effects
MH	Maleic hydrazide
MRM	Multiple reaction monitoring
PPP	Pyripropanol
PSA	Primary secondary amine
QuPPe	Quick polar pesticides

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RPLC	Reverse phase liquid chromatography
tZ	*trans*-Zeatin
UHPLC–QqQ-MS/MS	Ultrahigh-performance liquid chromatography – triple quadrupole tandem mass spectrometry
UV	Ultraviolet–visible

Introduction

Plant growth regulators (PGRs), which refer to the natural or synthetic chemicals showing similar physiological activity as endogenous plant hormones, affect versatile progresses in regulating plant life from sprouting, growth, maturity to reproduction, as well as response to the environmental stimuli [1–3]. The utilization of PGRs on foods aiming at increasing the production, such as crops [4–7], vegetables and fruits [3, 8], is necessary to feed the increasing population, from which human benefit a lot. An extensive use of PGRs on herbal medicine has been observed these years owning to the gap between supply and demand on the global market. The growth control on medicinal organ acting downstream of PGRs brings uncertainty to the internal quality of herbal medicine. Sharp decrease of principle active compounds after the indiscriminate and excessive use of PGRs in several Chinese herbs, especially for field cultivated root and rhizome herbs such as Ophiopogonis Radix and Platycodonis Radix, have been reported in recent research [9–11]. Many countries and regions have set maximum residue limits (MRLs) for certain PGRs in crops, vegetables and fruits [12–14]. Unlike the situation of foodstuffs, herbal medicine possesses proven pharmacological and synergistic therapeutic effects in various cultures [15–17]. To date, levels of PGRs in herbal medicines are lack of regularly monitoring and suitable standard to guide their use, thus the applicable scope and circumstances should be adequately addressed.

PGRs are a structurally unrelated collection of compounds, and a series of detection methods have been derived. Anastassiades et al. [18] introduced a QuEChERS method that was later modified by Lehotay et al. [19] to screen hundreds of pesticides, including PGRs, in food samples. Similar pretreatment procedures were also applied to herbal plant samples followed by the liquid chromatography (LC) coupling with different detectors including a fluorescence detector (FLD) [20, 21], diode array detector (DAD) [22, 23], and triple quadrupole tandem mass spectrometry (QqQ–MS/MS) analysis [3, 24, 25]. QuEChERS method works well for moderately polar and non-polar molecules that can be effectively partitioned with acetonitrile/water and retained on a reversed-phase column [26]. However, for very polar PGRs, QuEChERS method may not be a good choice because the insufficient quantitatively extract during the partitioning step. Zhao et al. [25] developed a PGR residue method in Ophiopogonis Radix, recoveries of choline chloride, one of the most used polar PGRs, was only 57.37%. European Reference Laboratory for Single Residue Methods (EURL-SRM) introduced an evolved residue analysis of high polar pesticides using acidified methanol, called QuPPe (Quick Polar Pesticides) [27]. This method allows for the simultaneous extraction of high polar pesticide residues and their metabolites from plant origin matrices with broad compatibility. However, methods for the multi-analysis of polar or mid-polar PGRs in herbal samples remain scarce.

In our preliminary study, 39 PGRs residues in Codonopsis Radix and Notoginseng Radix et Rhizoma were determined using QuEChERS method [24]. Taking the application frequency and amount into consideration, emphasis on the multiresidue of nine polar analytes, including benzimidazole, chlormequat chloride, diethylaminoethylhexanoate, daminozide, maleic hydrazide, mepiquat chloride, pyripropanol, *trans*-zeatin and fosamine ammonium, was thrown in this study. As the complementary method of the previous QuEChERS-based method, a modified QuPPe extraction procedure combined with low-temperature cleanup was further developed in three herbs, Isatidis Radix, Isatidis Folium and Platycodonis Radix. Isatidis Radix and Isatidis Folium are the dried root and leaf of *Isatis indigotica* Fort., which is known as Banlangen in China, are often used to treat influenza and other viral infectious diseases in clinic, as well as fever, dizziness, cough and sore throat [28]. Similarly, Platycodonis Radix is an ingredient in many traditional medicines that are clinically used in the relief of respiratory diseases such as bronchitis, asthma, tonsillitis, and pulmonary tuberculosis, and has been utilized at the forefront of the battle against COVID-19 in recent years [11, 29]. These three herbs are all traditional edible-medicinal species, which have been applied in many Asian counties for centuries. Analysis of 135 real samples was conducted thereof, including 40 Isatidis Radix samples, 42 Isatidis Folium samples and 53 batches of Platycodonis Radix.

Materials and Methods

Chemicals and Reagents

Analytical-grade standards benzimidazole (BNZ), chlormequat chloride (CCC), diethylaminoethylhexanoate (DA-6), daminozide (ALAR), maleic hydrazide (MH), mepiquat chloride (DPC), and fosamine ammonium (FA) were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Pyripropanol (PPP) and *trans*-zeatin (tZ) were obtained from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China) and Toronto Research Chemicals Inc. (Ontario, Canada), respectively. The isotopically labeled internal standard

Chlormequat chloride-*d*4 for positive ionization mode was got from Beijing Manhage Biotechnology Co., Ltd (Beijing, China). Detailed information of these analytes is given in Supplementary Table 1.

Acetonitrile and methanol, HPLC grade, were obtained from Avantor Performance Materials Inc. (Pennsylvania, USA). Formic acid and 10 M ammonium formate solution of LC/MS grade were purchased from Thermo Fisher Scientific Inc. (Shanghai, China) and Sigma-Aldrich Co. (St. Louis, USA), respectively. Sorbents of octadecylsilyl (C18), primary secondary amine (PSA), silica, Florisil, Alumina N (AL-N), graphitized carbon black (GCB) and ProElut PLS for dispersive solid-phase extraction were obtained from Beijing Dikma Technology Co., Ltd. (Beijing, China). Ultrapure water was generated by a Milli-Q ultra-pure water system (Milford, USA).

Plant Materials

135 herbal samples, provided by 54 TCM enterprises, were collected from typical cultivation area and local market of Shanghai, Anhui, Inner Mongolia, province in China (Supplementary Table 2). These herbs were ground into fine powder, and stored at –20 ℃ before use. The PGR contamination in herbal medicines might be inhomogeneous, so sampling should be representative.

Standard Preparation

Individual stock solutions of each PGRs standard and IS (1000 mg/L) were prepared in methanol, and then stored at –20 ℃ avoiding light. IS stock solution was further diluted into IS working solution with the concentration of 10 mg/L. Nine mixed PGRs standards at 100 μg/L and 1 mg/L (500 μg/L, 5 mg/L for MH and FA) were prepared as the fortification standards, which were used to fortify the herbal blank (5 g) at 10, 20, 100, 200, 400 and 800 μg/kg (fivefold concentration for MH and FA due to the lower sensitivity).

Sample Preparation Using the Optimized QuPPe Method

An aliquot of 5 g pulverized herbs was precisely weighed each in a 50 mL polypropylene centrifuge tube, and 20 mL 50% methanol (1% formic acid) and 200 μL IS working solution were pooled together. The tube was capped and shaken horizontally for 5 min at the rate of 500 times per minute on an IKA KS 260 basic shaker (Staufen, Germany). The tube was then placed in a Haier DW-86L386 ultralow-temperature freezer (Qingdao, China) at –80 ℃ for 30 min, and instantly centrifuged for 5 min at 4000 rpm (Eppendorf Centrifuge 5920R, Hamburg, Germany). 1.5 mL supernatant was transferred to a 2 mL centrifuge tube, which contained 100 mg C18, 50 mg silica and 5 mg GCB for the *d*-SPE cleanup. Shaken vigorously for another 5 min and centrifuged for 5 min at 14,000 rpm (Eppendorf Centrifuge 5424R, Hamburg, Germany). 1.0 mL of the supernatant was filtered through a 0.22 μm PTFE filter (Dikma, Beijing, China) for further analysis by UHPLC–MS/MS. Wherein matrix matched internal standard method was applied for the quantification of analytes under positive ionization mode, and matrix matched external standard method for FA under negative ionization mode.

Chromatographic and Mass Spectrometric Conditions

A triple quadrupole mass spectrometer system (6500 + , AB Sciex Instruments, Concord, Canada) equipped with an electrospray ion (ESI) source, coupled to an LC system (Shimadzu, Kyoto, Japan), consisting of two Nexera X2 LC-30AD LC-pumps, a SIL-30ACMP autosampler and a CTO-20AC column oven, was used for method development and validation. A hydrophilic interaction liquid chromatography (HILIC) analytical column (XBridge BEH Amide, 2.5 μm, 100 × 3.0 mm) from Waters Co., Ltd. (Dublin, Ireland) was used for LC separation thermostated at 35 ℃ with a sample injection volume of 2 μL. The mobile phase consisted of 50 mmol ammonium formate (pH adjusted to 3 with formic acid, (A) and acetonitrile (B) with the flow rate of 0.4 mL/min. The linear gradient elution program started at 97% B for 0–0.5 min, 97–70% B for 0.5–4 min, 70–50% B for 4–5 min, 50% B for 5–6 min, 97% B for 6–6.1 min and 97% B for 6.1–10 min.

The MS determination was operated in the multiple reaction monitoring (MRM) mode with polarity switching of positive and negative ionization. The ESI source conditions were as follows: curtain gas (CUR) of 275.80 kPa, ion spray voltage (ISV) of 5500 V (pos) and –4500 V (neg), nebulizer gas (GS1) of 344.75 kPa, heater gas (GS2) of 379.23 kPa, and source temperature of 500 ℃.

Analytical Performance Parameters

The method will be validated in three herbal medicines, which are Isatidis Radix, Isatidis Folium and Platycodonis Radix. The method validation performance criteria is evaluated by assessing the linearity of the calibration curve, matrix effects, trueness (as % recovery), precision (as repeatability % RSDs) and method quantitation limits (LOQ), following the EU SANTE Guideline on analytical quality control and validation procedures [30].

Matrix effects (MEs) are measured by the equation: MEs (%) = slope $_{matrix}$/slope $_{solvent}$ × 100%, where slope $_{matrix}$ is the slope of matrix-based calibration, and slope $_{solvent}$ is the slope of solvent-based curve with the same prepared

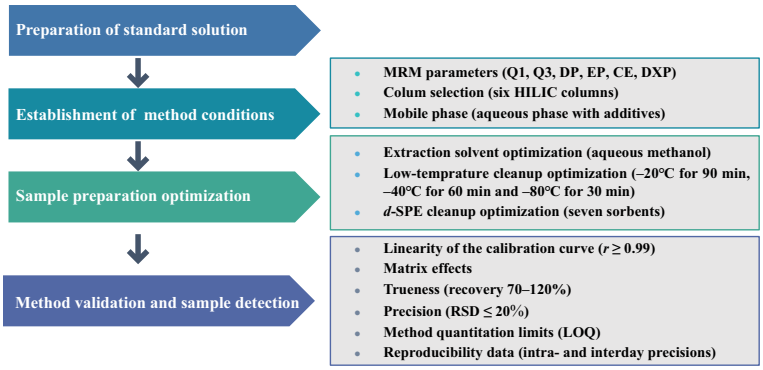

Fig. 1 The schematic diagram of the designed experiment

concentration. MEs < 100% means ionization suppression, while MEs > 100% suggests ionization enhancement.

Four spiking levels with six replicates ($n = 6$) will be adopted to determine the trueness (as % recovery) and precision (as % RSD, $n = 6$) in the recovery experiments. Reproducibility expressed as intra- and inter-day precisions will be obtained using replicated spiking herbal sample in routine analysis series (within one day) and on three consecutive days, respectively. The method LOQ refers to the lowest spiking level in recovery experiment that can be quantified with acceptable trueness (recovery, 70–120%) and precision (RSD ≤ 20%). The schematic diagram of the designed experiment is shown in Fig. 1.

Results and Discussion

Liquid Chromatography Optimization

Single standard solution at 1 mg/L in methanol for each analyte was directly infused into the mass spectrometer to obtain optimal conditions of Q1 and Q3 MRM transitions, declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP). Full scans in ESI + and ESI– ionization mode were conducted, and result showed that all PGRs and IS analytes, except for FA, tended to render abundant [M + H] + ions in ESI + mode. While for FA, the stable $[M–NH4]^-$ ion could be produced with good response in ESI– mode. These MS parameters are shown in Supplementary Table 3.

As recommended by European Union reference laboratory for single-residue methods (EURL-SRM) [30], properties of six different HILIC analytical columns were compared for the polar and moderately polar analytes. The column of Waters XBridge BEH Amide (c, 2.5 µm, 100 × 3.0 mm) was chosen ultimately, and the others used in the pre-experiments were Agilent Poroshell 120 HILIC-Z (a, 2.7 µm, 100 × 2.1 mm), Waters CORTECS UPLC HILIC (b, 1.6 µm, 150 × 2.1 mm), Waters ACQUITY UPLC BEH Amide (d, 1.7 µm, 100 × 3.0 mm), Phenomenex Kinetex HILIC (e, 1.7 µm, 100 × 2.1 mm) and Waters XBridge Amide (f, 3.5 µm, 100 × 2.1 mm) in consideration of different particle technologies and bonding functions. Columns a and f possessed weaker retain towards these polar analytes. As the typical solid-core particle designed column, column b showed worse separation in

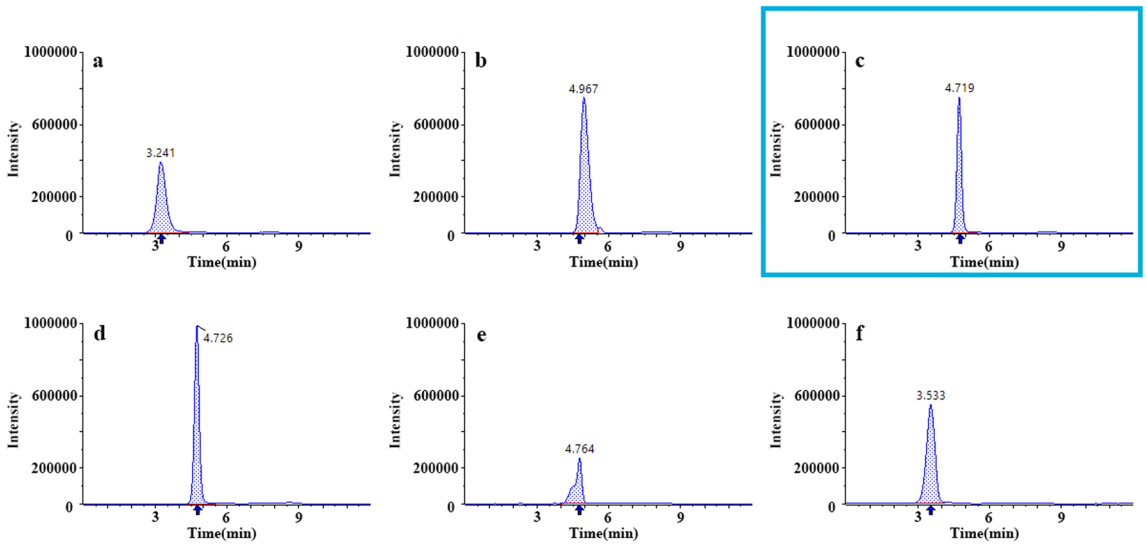

Fig. 2 MRM chromatograms of ALAR in standard solvent (100 µg/L, 1 µL) on HILIC column **a**–**f**

terms of peak tailing. Peak shape of ALAR was poor on column e, as shown in Fig. 2. Though column d showed comparative separation with c, the 2.5 µm particle size enabled exceptional separation performance, robustness and throughput for HPLC assays while enabling a seamless transition path toward UPLC adoption. The proposed approach decreased the matrix suppression for LC–MS/MS detection by avoiding the elution close to the solvent front, and improved the poor chromatographic peak shape. TIC chromatograms of the investigated columns could be checked in Supplementary Fig. 1.

Acetonitrile, the aprotic medium, is commonly used in the HILIC separation system owning to its reinforcement effect of hydrogen bonding between the polar adsorption layer on the surface of analyte and stationary phase [31, 32]. Aqueous phase of different pH was investigated, including pure water, 10 mmol ammonium formate (pH adjusted to 3 with formic acid), 50 mmol ammonium formate (pH adjusted to 3 with formic acid) and 50 mmol

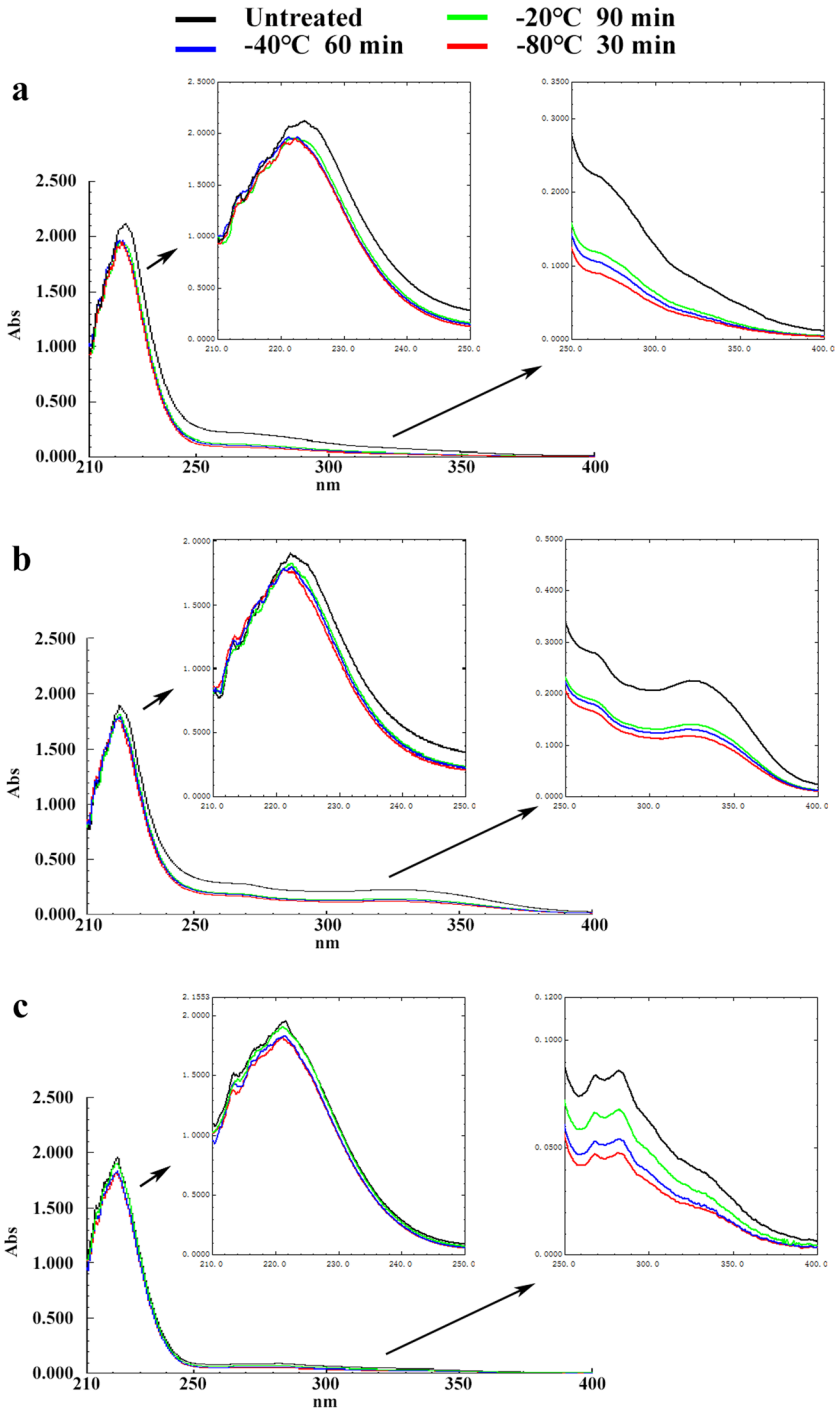

Fig. 3 Cleanup efficiency evaluated by UV scanning under different low-temperature incubations for Isatidis Radix (**a**), Isatidis Folium (**b**) and Platycodonis Radix (**c**)

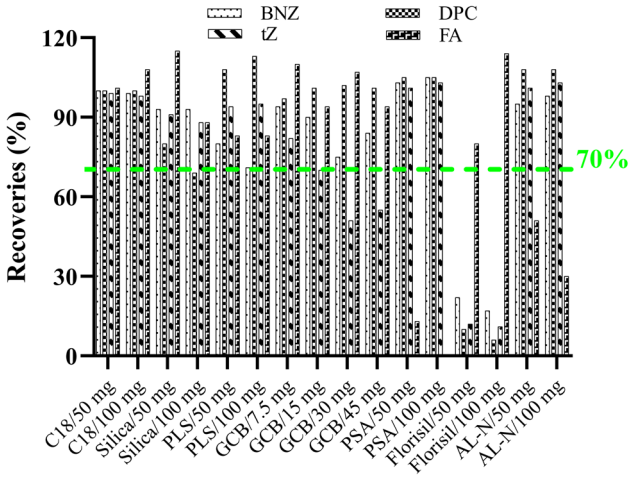

Fig. 4 Effects of single sorbent on the mean recoveries of key target compounds ($n = 2$)

ammonium formate (pH 6.8). Obvious tailed CCC peak was observed when using pure water as the mobile phase, which could be rescued by the additives of appropriate concentration such as ammonium formate and formic acid. 50 mmol ammonium formate (pH 6.8) tended to render a bad shape of ALAR peak. Good separation and sensitivity were obtained under the aqueous phase of 10 mmol ammonium formate (pH 3) and 50 mmol ammonium formate (pH 3), while the latter condition showed better MS response probably because the ionization improvement with the increased concentration of buffer salt in the mobile phase. Thus, 50 mmol ammonium formate (pH 3) worked as the aqueous phase in this study.

Sample Extraction Optimization

The optimization of the extraction solvent was performed by comparing the recoveries of nine PGRs at the spiking level of 50 µg/L in three blank herbal matrices based on QuPPe-based method. The tested extraction solvents covered 10, 30, 50 and 70% aqueous methanol containing 1% formic acid. Results showed that 10, 30 and 50% acidic aqueous methanol could reach the recovery requirement between 70 and 120%. Nevertheless, excessive water ratio froze easily at cold temperature, thus 50% acidic aqueous methanol was adopted as the extraction solvent.

Optimization of Low-temperature Cleanup

Low-temperature cleanup was applied in the detection of trace contaminants, such as endocrine disruptors [33], veterinary drugs, mycotoxins and pesticides [34–36]. This procedure showed good preliminary purification towards oil-rich food and complex matrices like spices and herbs, without influencing the recovery of analytes from the matrix. Low-temperature cleanup was also reaccommodated in general principle of 2341, Chinese Pharmacopoeia 2020 edition, recording that a 10-min ice bath was required after the salting out process [37]. At low temperature, the impurities in herbal samples solidified and flocculated which was then removed through centrifugation.

In this study, different low-temperature incubation conditions were investigated, which included –20 ℃ for 90 min, –40 ℃ for 60 min and –80 ℃ for 30 min. Appearances of the 50% methanol extracts were first assessed visually. The color of the low-temperature incubated extracts was obviously lighter compared with the untreated ones, which was exhibited in Supplementary Fig. 2. The cleanup efficiency was further evaluated by the ultraviolet–visible (UV) scanning in 210–400 nm with an UV spectrophotometer (Agilent Cary Series UV–Vis, Agilent Technologies, Santa Clara, CA, USA), and lower absorbance represented better purification. As displayed in Fig. 3, absorbance dropped particularly in the range of 250–400 nm for all the tested herbal samples, which might suggest the decreased matrix effects. Best cleanup effect could be achieved at –80 ℃ in a much shorter time. This low-temperature incubation was utilized before the *d*-SPE cleanup.

Optimization of d-SPE Cleanup

Seven dispersive sorbents for the subsequent cleanup step were separately tested. These sorbents possessed multifunctional adsorption of nonpolar interferences like fatty acids (C18), polar interferences like sugar (PSA, silica, Florisil and AL-N), pigments (GCB) and common substances adsorbed by hydrophilic–lipophilic balanced ProElut PLS, as adequately addressed in numerous literature [38, 39]. 1.5 mL mixed standard in 50% methanol (100 µg/L) was precisely measured in a 2-mL tube. Single sorbent was weighed into, prior the combined purification, with the described amounts: 50/100 mg C18, 50/100 mg silica, 50/100 mg PLS, 7.5/15/30/45 mg GCB, 50/100 mg PSA, 50/100 mg Florisil and 50/100 mg AL-N. Shaken vigorously for 5 min and centrifuged for 5 min at 14,000 rmp. 1.0 mL of the supernatant was analyzed, and the recoveries were compared. In our study, Florisil was not applicable due to the lowest recoveries for most of the analytes. Recoveries of FA decreased significantly with the increased amounts of PSA and AL-N. PGRs of aromatic structure like tZ tended to be absorbed by GCB; however, the adsorption was acceptable within reasonable amounts. Effect of single sorbent on the recoveries of BNZ, DPC, tZ under ESI + mode and FA under ESI– is illustrated in Fig. 4. Herein, C18, silica, PLS

and GCB were integrally evaluated to verify the combined purification capacities.

The combination of 100 mg C18 + 50 mg silica, 100 mg C18 + 50 mg silica + 5 mg GCB, 100 mg C18 + 50 mg silica + 10 mg GCB, 100 mg C18 + 50 mg PLS, 100 mg C18 + 50 mg PLS + 5 mg GCB and 100 mg C18 + 50 mg PLS + 10 mg GCB were evaluated. Besides, the classic SPE approach using Oasis PRiME HLB 6 cc (200 mg) Extraction Cartridges (Waters Co., Ltd., Milford, MA, USA) [34] was also compared. Recoveries of 1.5 mL 100 μg/kg spiked level in three herbal matrices were used to measure the purification efficiencies. It was obviously to see that PLS showed affinity towards DA-6, which brought on the decrease in its recoveries in Fig. 5. Combined purification was equivalent, and even prevailed HLB SPE when the dosage of GCB was controlled at 5 mg. The final *d*-SPE sorbent types and amounts were clarified to be 100 mg C18, 50 mg silica and 5 mg GCB.

The synergistic cleanup effect of –80 ℃ low-temperature incubation and *d*-SPE was visualized via the UV scan on Agilent 1290 UHPLC (Agilent Technologies, Santa Clara, CA, USA) in the range of 210–300 nm. The decline in the UV absorbance was observed for Isatidis Radix, Isatidis Folium and Platycodonis Radix extract (Fig. 6). Despite

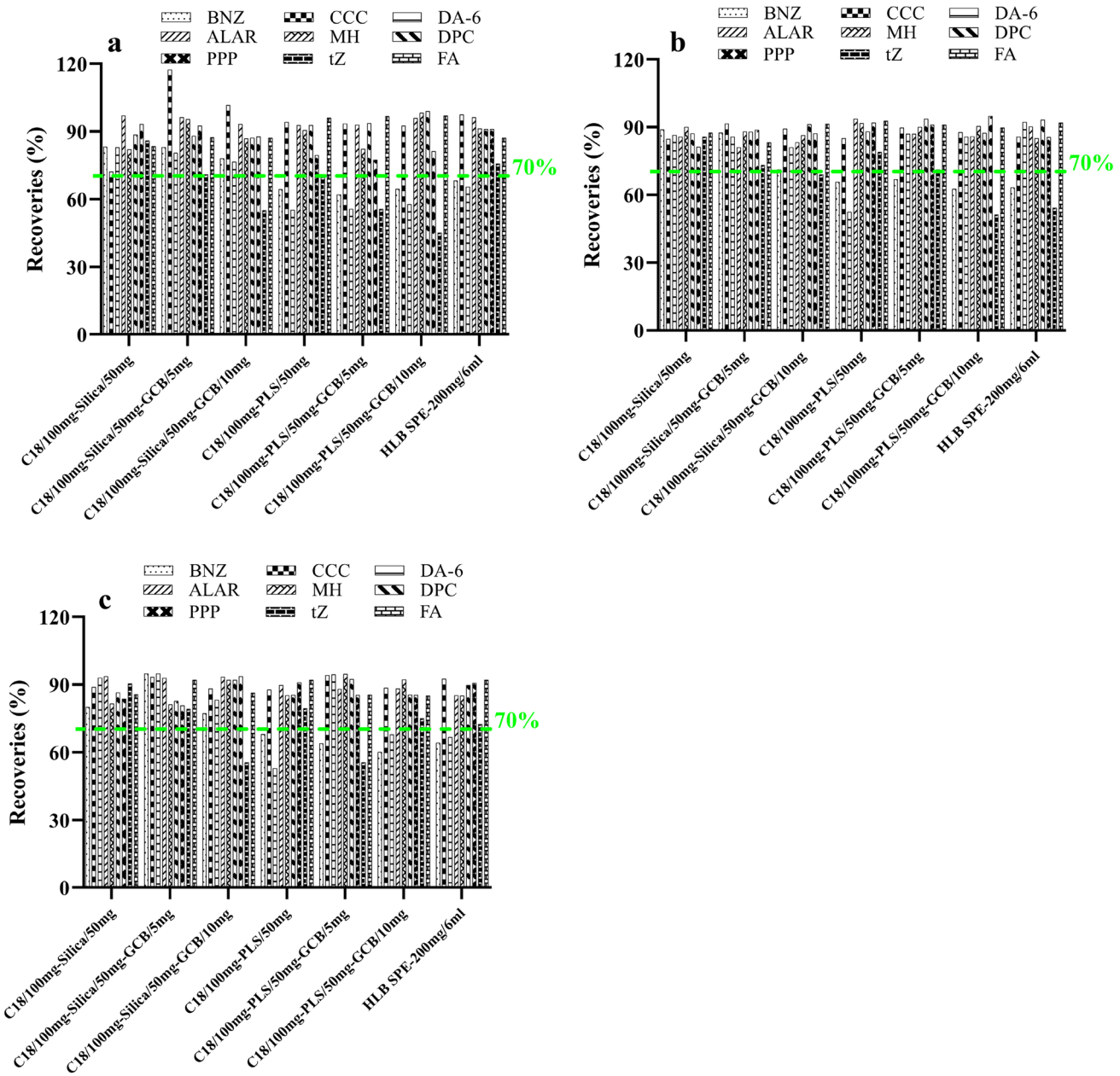

Fig. 5 Efficiency of combined sorbents and HLB SPE on the mean recoveries of nine target compounds in Isatidis Radix (**a**), Isatidis Folium (**b**) and Platycodonis Radix (**c**) ($n = 2$)

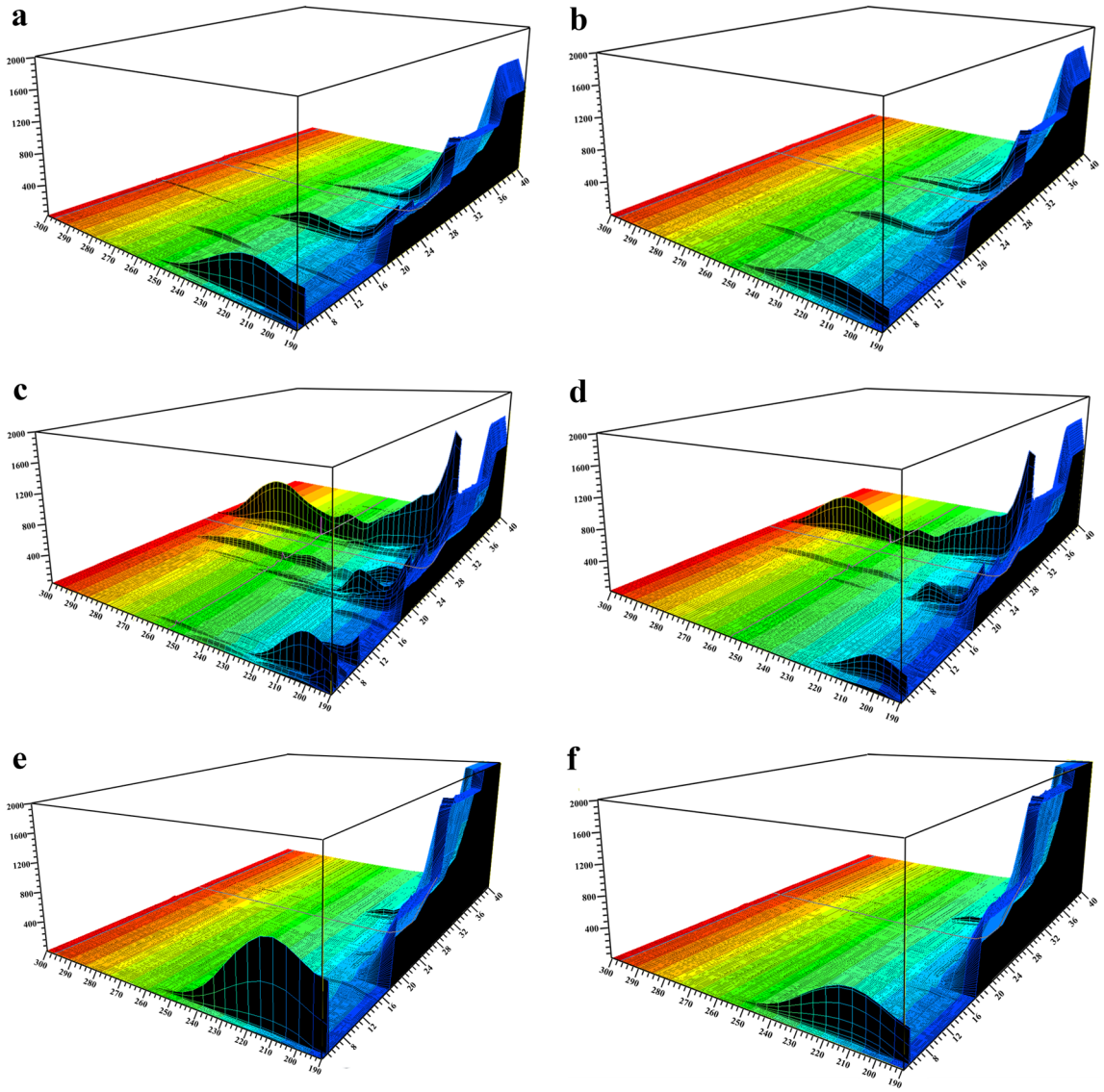

Fig. 6 Synergistic cleanup efficiency evaluated by UV scanning for untreated Isatidis Radix extract (**a**), Isatidis Folium extract (**c**) and Platycodonis Radix extract (**e**); purified Isatidis Radix extrct (**b**), Isatidis Folium extract (**d**) and Platycodonis Radix extract (**f**)

the advances in the development of analytical instrumentation for determination of various contaminants, the sample pretreatment is a determinant step to obtaining satisfactory results [40, 41]. One of the most important factors that make the multiresidue determination of PGRs in herbal matrices challenging is the co-elution of complex matrices. Here in this study a potential approach consisting of low-temperature cleanup and *d*-SPE could be provided.

Method Validation

Linearity, LOQ and MEs

The absence of background peaks at the retention times of the target analytes showed that no interference occurred. Calibration curves were constructed with six concentration levels from 2.5 to 200 μg/L, which was corresponding to 10–800 μg/kg. The levels for MH and FA were five times higher owning to lower sensitivities. Six-point matrix matched calibration curves, with the weighting factor of *1/x*, were established with correlation coefficients appraised by *r*. Linearities were satisfying with all the correlation coefficients higher than 0.99, as shown in Table 1. The criterion of a maximum deviation of 20% was met in all cases. MEs brought on by the presence of co-extraction interferences were calculated and results found that unignorable ionization suppression existed during the detection of these polar PGRs in herbal medicines. MEs in Isatidis Folium for tZ were as

significant as 13%. The importance of the matrix matched internal standard method stands out for the accuracy.

Recoveries and Reproducibility

Recoveries were presented as mean percentage of six replicates at 1 × LOQ, 2 × LOQ, 10 × LOQ and 20 × LOQ spiking levels with blank matrices of Isatidis Radix, Isatidis Folium and Platycodonis Radix. Average recoveries for all the analytes ranged from 64.6% to 117.8%, and RSDs were within 1.2–16.8%. Satisfactory results were obtained for most of the investigated PGRs according to the criteria from the EU SANTE Guideline (Table 2), except for DA-6 in Platycodonis Radix. Recoveries were not suitable with the values of 64.6% (0.2 mg/kg) and 66.4% (0.4 mg/kg). Despite this, the optimized method showed robustness and could be applied to the determination of polar PGRs in herbal samples. Intra- and inter-day precisions timely acquired were compliance to the validation requirement with RSDs below 20%.

Positive Rate of Target Polar PGRs

It is found that most agricultural fertilizers labeled as water-soluble fertilizers containing nitrogen, phosphorus, potassium and essential elements were doped with multiple kinds of polar PGRs without marking [42]. Hence, their actual application amount might be beyond our comprehension. Although human benefit from the increased production of agricultural products, the use of polar PGRs also put us into the situation of environmental pollution and food poisoning [43–45]. The poor liquid–liquid extraction and quick elution on reverse phase liquid chromatography (RPLC) system makes the detection of polar PGRs easy to be ignored. The established method aiming at the full exposure of polar PGRs was applied to 135 herbal samples, including 40 batches of Isatidis Radix, 42 batches of Isatidis Folium and 53 batches of Platycodonis Radix. The occurrence is summarized in Table 3. The overall positive rate for these nine analytes was 17.8%, wherein CCC and DPC were the top two polar PGRs of highest detection frequency that were investigated in this study. Positive rate for CCC was 15.6%, of which the detected concentration ranged from 11.15 to

Table 1 Linear range, LOQ and MEs for Isatidis Radix, Isatidis Folium and Platycodonis Radix

Analyte	Sample	Linear range (μg/L)	Linear equation	*r*	LOQ (μg/kg)	MEs (%)
BNZ	Isatidis radix	2.5–200	$y = 0.13984\ x + 0.39563$	0.999	10	53
	Isatidis folium	2.5–200	$y = 0.13488\ x + 0.63000$	0.999	10	49
	Platycodonis radix	2.5–200	$y = 0.15534\ x + 0.64412$	0.996	10	60
CCC	Isatidis radix	2.5–200	$y = 0.02674\ x + 0.81827$	0.997	10	32
	Isatidis folium	2.5–200	$y = 0.02102\ x + 0.66378$	0.999	10	26
	Platycodonis radix	2.5–200	$y = 0.03968\ x + 2.19074$	0.998	10	52
DA-6	Isatidis radix	2.5–200	$y = 0.04510\ x + 0.01002$	0.999	10	40
	Isatidis folium	2.5–200	$y = 0.04619\ x + 0.01696$	0.999	10	39
	Platycodonis radix	2.5–200	$y = 0.06008\ x + 0.01571$	0.999	10	54
ALAR	Isatidis radix	2.5–200	$y = 0.01705\ x + 0.02481$	0.999	10	56
	Isatidis folium	2.5–200	$y = 0.01640\ x + 0.10920$	0.999	10	52
	Platycodonis radix	2.5–200	$y = 0.01996\ x + 0.02270$	0.999	10	67
MH	Isatidis radix	12.5–1000	$y = 4.42048e\text{-}4\ x + 0.00111$	0.996	50	35
	Isatidis folium	12.5–1000	$y = 5.86503e\text{-}4\ x + 8.89531e\text{-}4$	0.999	50	42
	Platycodonis radix	12.5–1000	$y = 6.54523e\text{-}4\ x + 3.93955e\text{-}4$	0.996	50	57
DPC	Isatidis radix	2.5–200	$y = 0.08616\ x + 0.06320$	0.999	10	43
	Isatidis folium	2.5–200	$y = 0.09469\ x + 0.15469$	0.998	10	45
	Platycodonis radix	2.5–200	$y = 0.11657\ x + 0.05511$	0.997	10	59
PPP	Isatidis radix	2.5–200	$y = 0.03191\ x + 0.02278$	0.999	10	51
	Isatidis folium	2.5–200	$y = 0.03063\ x + 0.05051$	0.999	10	47
	Platycodonis radix	2.5–200	$y = 0.03931\ x + 0.03715$	0.997	10	65
tZ	Isatidis radix	2.5–200	$y = 0.05042\ x + 0.04898$	0.999	10	33
	Isatidis folium	2.5–200	$y = 0.02105\ x + 0.04728$	0.999	10	13
	Platycodonis radix	2.5–200	$y = 0.08190\ x + 0.07417$	0.997	10	53
FA	Isatidis radix	12.5–1000	$y = 384.33444\ x + 365.42183$	0.998	50	23
	Isatidis folium	12.5–1000	$y = 289.64249\ x + 285.20321$	0.999	50	52
	Platycodonis radix	12.5–1000	$y = 924.01354\ x + 146.45148$	0.996	50	67

Table 2 Recoveries and RSDs ($n=6$)

Analyte	Spike levels (mg/kg)	Isatidis radix		Isatidis folium		Platycodonis radix	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
BNZ	0.01	89.2	12.9	73.2	5.7	73.7	4.5
	0.02	95.7	4.9	98.9	16.8	77.7	6.5
	0.1	94.7	4.3	79.1	3.6	93.6	2.4
	0.2	91.4	2.3	82.4	5.5	94.8	4.3
CCC	0.01	94.6	2.9	98.2	3.1	99.5	2.7
	0.02	93.5	2.9	99.5	2.4	99.9	1.4
	0.1	91.8	1.6	96.7	2.4	99.0	5.0
	0.2	90.8	1.2	97.9	2.7	99.8	1.2
DA-6	0.01	79.1	7.8	74.9	4.3	74.3	4.1
	0.02	86.4	8.0	80.1	3.1	76.2	3.7
	0.1	101.1	4.3	81.5	4.5	64.6	1.9
	0.2	95.4	3.1	81.5	2.1	66.4	4.6
ALAR	0.01	105.9	11.8	84.6	10.4	95.6	13.7
	0.02	106.7	7.1	101.6	15.9	103.3	4.5
	0.1	109.3	5.6	94.8	6.7	103.6	2.1
	0.2	117.5	2.6	97.4	5.8	105.6	4.2
MH	0.05	87.0	3.9	94.3	13.1	104.5	13.1
	0.1	97.3	7.5	86.1	16.6	105.9	5.4
	0.5	115.4	12.7	85.4	6.5	100.9	5.8
	1	115.3	5.6	81.4	5.7	101.7	7.2
DPC	0.01	90.5	7.0	71.4	4.6	89.5	7.2
	0.02	101.1	4.2	89.3	4.5	96.3	4.1
	0.1	117.8	2.9	92.5	4.2	94.0	3.8
	0.2	112.7	4.1	90.9	6.1	96.5	3.6
PPP	0.01	104.1	9.4	85.0	5.6	97.9	9.7
	0.02	106.5	6.2	94.5	4.7	102.7	5.1
	0.1	107.9	4.9	92.1	2.7	102.0	2.3
	0.2	104.1	2.1	93.4	5.7	103.1	4.6
tZ	0.01	70.7	12.2	71.1	2.5	73.2	3.5
	0.02	86.5	13.3	71.4	9.2	72.8	5.3
	0.1	105.3	10.9	75.4	4.7	73.4	2.4
	0.2	99.4	2.4	77.0	5.0	75.9	3.6
FA	0.05	83.5	10.9	84.2	5.7	93.4	11.4
	0.1	85.2	6.4	88.7	16.6	86.9	4.0
	0.5	94.3	5.2	94.4	3.7	81.1	10.8
	1	87.7	10.7	91.6	8.1	79.5	4.6

373.89 μg/kg. As recorded in GB 2763–2021 released in China [14], the MRLs for CCC is below 5 mg/kg in wheat and 1 mg/kg in tomato. Nevertheless, Commission Regulation (EU) 2022/1290 of 22 July 2022 amended Regulation (EC) No 396/2005 as regard to maximum residue levels for CCC in tea to be below 0.05 mg/kg (https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=uriserv%3AOJ.L_.2022.196.01.0074.01.ENG&toc=OJ%3AL%3A2022%3A196%3ATOC, accessed on February 3, 2023), which indicates that the limit levels evaluation of PGRs is always under concern around the world.

It is important to point out that the risk of multi-contamination is still high, according to the repeated and extensive application of those functional chemicals [42, 46]. Moreover, Isatidis Radix, Isatidis Folium and Platycodonis Radix are medicine food homology species, which most likely means people consume them with larger amount in a long-term pattern. Our team has been devoting on improving the detection system of exogenous toxic and harmful substances these years for the reliable contamination data in traditional herbs. The applicability and performance of the built methods have been evolving. The method for the multi-residue

Table 3 Positives of 9 polar PGRs in 135 herbal samples

Herb name	Samples	Positives	Detection range (μg/kg) (positives)	
			CCC	DPC
Isatidis radix	40	5	12.61–339.60 (3)	47.17–176.70 (2)
Isatidis folium	42	7	17.97–134.95 (6)	43.20 (1)
Platycodonis Radix	53	12	11.15–373.89 (12)	/[a]
Total number	135	24	21	3

[a]indicates not detected or < LOQ

analysis of 39 PGRs was optimized and developed in our preliminary study based on RPLC system via QuEChERS extraction [24]. Individualized study of polar PGRs based on HILIC system via QuPPe extraction was followed up in regard of their bad extraction efficiency and chromatographic behavior. The establishment of the integrated detection system is conducive to the scientific accumulation of more PGR residual data, and thus helps supervising the PGR utilization in herbal medicines.

Conclusions

In the present study, a liquid chromatography–tandem mass spectrometry method based on QuPPe extraction was developed for the detection and quantification of the residues of nine widely used polar PGRs. Sample pretreatment of low-temperature incubation followed by *d*-SPE cleanup process, HILIC chromatographic separation, as well as validation in herbal matrices of Isatidis Radix, Isatidis Folium and Platycodonis Radix based on EU SANTE/12682/2019 Guideline were intensively discussed. 135 batches of herbs were analyzed thereof. Validation and data collection covering more herbal matrices will be carried out under consideration with the aim of full risk characterization of PGRs.

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Data Availability The authors confirm that the data supporting the findings of this study are available within the article, and the supplementary materials.

Declarations

Conflict of interest The authors declare no conflict of competing financial interests.

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