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Contents of flavonoid compounds in *Dendrobium officinale* Kimura et Migo determined by QuEChERS-HPLC-MS/MS: Method validation and influencing factors

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ABSTRACT

Dendrobium officinale Kimura et Migo (*D. officinale*), a dual-use plant with both botanical medicine and food applications, has gained global popularity. Flavonoids are important bioactive compounds of *D. officinale*; nevertheless, the quantitative analysis method for flavonoid components is lack, restricting an in-depth understanding of their functions. A QuEChERS method coupled with HPLC-MS/MS was developed to detect 17 flavonoid components of *D. officinale*. Octadecylsilane was selected as the clean-up sorbent, which exhibited a relatively weak affinity for flavonoids yet an effective removal of pigments from extracts. Method validation showed that limits of detection (LODs) and limits of quantification (LOQs) were 2.1–49.5 and 7.1–165.2 µg/kg, respectively. The recoveries ranged from 73 % to 112 %, with inter-day and intra-day RSDs below 20 %. Among the 17 flavonoids measured, naringenin, rutin and schaftoside were the predominant compounds, with the highest values of 57.18, 563.17 and 67.73 mg/kg, respectively. These three flavonoids accounted for over 80 % of the total content of 17 flavonoids. Moreover, the Multiple Factor Analysis indicated that the origin and cultivation method exerted great influence on the flavonoid concentrations in *D. officinale*. The results have important implications for further research and utilization of flavonoids in *D. officinale*.

1. Introduction

Dendrobium officinale Kimura et Migo (*D. officinale*) has been traditionally applied in folk medicine in China and Southeast Asian countries for more than 2000 years (Guo et al., 2021). It shows antioxidant, anti-tumor and immune-enhancing effects, and is increasingly used in pharmaceutical, health food and cosmetic industries (Huanget al., 2016;

Luo et al., 2016; Chanet et al., 2018; Meng et al., 2019). In 2019, *D. officinale* was listed as a dual-use plant for botanical medicine and food applications by the National Health Commission of the People's Republic of China (National Health Commission of the People's Republic of China, 2020). *D. officinale* has become widely popular not only in the Chinese market, but also spread to many other countries, such as Australia, Japan and the United States (Xianget al., 2013). In the first

Abbreviations: *D. officinale*, *Dendrobium officinale* Kimura et Migo; LOD, Limit of detection; LOQ, Limit of quantification; QuEChERS, Quick, Easy, Cheap, Effective, Rugged, Safe; C18, Octadecylsilane; PSA, Primary secondary amine; g-MWCNTs, Graphitized multi-wall carbon nanotubes; MWCNTs-OH, Hydroxylated multi-wall carbon nanotubes; MWCNTs-COOH, Carboxylated multi-wall carbon nanotubes; MFA, Multiple Factor Analysis; DPPH, 2,2-diphenyl-1-picrylhydrazyl; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; ABTS, 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate); ESI, Electrospray ionization; MRM, Multiple reaction monitoring; Ct, Total content of flavonoid; CE, Collision energy; Rf, Concentration ratio of flavonoid component in the extract before and after purification. Peer review under responsibility of King Saud University.

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half of 2020, 13 tons of *D. officinale* were exported with a total value of 12.08 million US dollars (Fu et al., 2021).

The antioxidant activity is one of the most significant biological functions of *D. officinale* (Yuan et al., 2020), which is closely related to contents and types of flavonoids (Yuan et al., 2020; Wan et al., 2021; Ma et al., 2023). The determination of concentrations and compositions of flavonoids is fundamental to clarify their mechanism of action. The total content of flavonoids in foods and herbs is generally determined by the sodium nitrite-aluminum nitrate spectrophotometry method (Xue et al., 2013; Zhang et al., 2017). Though this method is simple and convenient, it cannot provide information on the compositions of flavonoids, thus restricting research on their functions. Recently, some studies have focused on determination of flavonoid compositions and concentrations by using LC-MS (Ye et al., 2017; Zhou et al., 2018; Kim et al., 2023). In these works, *D. officinale* samples were extracted by methanol and then directly measured on LC-MS without sample clean-up. Nevertheless, *D. officinale* is a complex matrix, containing numerous substances such as pigments, polysaccharides, bibenzyls and alkaloids (Tang et al., 2017). These co-extracts would reduce the column efficiency and cause deterioration of chromatographic peak shape. Hence, the purification of extracts is essential to measure flavonoid compounds in *D. officinale* by using LC-MS. The QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) method is a widely used sample extraction and purification technique in the analysis of pesticide residues in agricultural products (Garcia-Vara et al., 2022). It shows advantages of simplicity, speed, cost-effectiveness and time-saving compared to traditional extraction techniques, such as matrix solid-phase dispersion or solid-phase extraction (SPE). The procedure includes sample homogenisation, solvent extraction/partitioning (with a salting-out process), clean-up through a dispersive SPE (dSPE) by using combinations of sorbents and salts, and analysis (Garcia-Vara et al., 2022). Potentially, the QuEChERS method could be used in the detection of bioactive compounds in foods. Recently, it has been modified for the determination of phenolic compounds in fruits and vegetables (Aguirret et al., 2020; Nicácio et al., 2020). However, the application of the QuEChERS method to detect flavonoid compounds of *D. officinale* has not been reported.

The accumulation of bioactive compounds is influenced by geographical location, cultivation environment and growth stage (Zhou et al., 2018; Gai et al., 2019). *D. officinale* is mainly distributed in Zhejiang, Yunnan, Guangdong, Anhui and Guangxi Provinces (Zhou et al., 2018). Flavonol contents of *D. officinale* grown in Guangdong Province were reported to be higher than those grown in Zhejiang and Guangxi Provinces (Lan et al., 2022). The cultivation methods of *D. officinale* include facility-aided cultivation and imitative wild cultivation, and the available products in the market are derived from various processing techniques, including Fengdou and dry stems. However, it remains unclear how cultivation method, growth years, collecting time and processing techniques impact the content and composition of flavonoid compounds.

In this study, the QuEChERS method was applied in quantitative analysis of flavonoid components in *D. officinale*. The goals of the current study were: i) to develop a QuEChERS method in combination with HPLC-MS/MS for determining 17 flavonoid components in *D. officinale*. The efficacy of five sorbents for sample clean-up including octadecylsilane (C18), primary secondary amine (PSA), graphitized multi-wall carbon nanotubes (g-MWCNTs), hydroxylated multi-wall carbon nanotubes (MWCNTs-OH), carboxylated multi-wall carbon nanotubes (MWCNTs-COOH) were explored and compared; ii) to determine the levels of 17 flavonoid components in 47 *D. officinale* samples by using the validated method. Furthermore, the Multiple Factor Analysis (MFA) was performed to scrutinize the relationship between flavonoid contents and factors such as origin, cultivation method, growth years, collecting time and processing; iii) finally, the antioxidant activities of the extracts were examined by the DPPH radical scavenging activity assay, ABTS radical scavenging activity assay and FRAP assay.

2. Materials and methods

2.1. Chemicals and reagents

HPLC-grade of methanol, acetonitrile, ammonium acetate and formic acid were obtained from Merck (Darmstadt, Germany). Analytical grade of NaNO_2 , $\text{Al}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ and NaOH were purchased from Macklin (Shanghai, China). Analytical grade of acetic acid, potassium persulfate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) and ferric chloride sodium acetate were supplied by J&K (Beijing, China). Sorbents PSA and C18 were provided by Agilent Technologies (Tianjin, China). g-MWCNTs (outer diameter: 10–20 nm, length: 5–30 μm), MWCNTs-COOH (outer diameter: 10–20 nm, length: 10–30 μm) and MWCNTs-OH (outer diameter: 10–20 nm, length: 5–30 μm) were acquired from XFNANO (Nanjing, China). Flavonoid standards including naringenin, apigenin, cynaroside, quercetin, eriodictyol, isorhamnetin, quercitrin, rutin, hesperetin, isoquercitrin, kaempferol, apiin, hyperoside, schaftoside, chrysoeriol, taxifolin and naringin were purchased from Anpu (Shanghai, China), with purities ranging from 97.1 % to 99.9 %. The structure of flavonoid compounds was listed in Table S1. Standard stock solutions of all flavonoid compounds (100 mg/L) were prepared by using methanol and stored at 4 °C until use.

2.2. Sample collection and pretreatment

Forty-seven samples of *D. officinale* were collected from production bases and markets. Detailed sample information including origin, cultivation method, growth years, collecting time, and processing was recorded in Table S2. Samples were cultivated under facility-aided cultivation mode or imitative wild cultivation mode. Fresh stems were processed into Fengdou, dry stems and slices. Generally, *D. officinale* of two-year-old was suggested to be harvested and sold in the market, and *D. officinale* of more than two-year-old were also collected to explore the effect of growth years on flavonoid contents. *D. officinale* was normally harvested during November to May of next year and *D. officinale* was also collected in non-harvest period to investigate the effect of collecting time on flavonoid contents. When fresh stems were collected, they were cut into small sections and then roasted using a DHG-9070A blast dryer (Shanghai Jinghong Experimental Equipment Co. LTD, China) for 24 h at 80 °C, following the same baking conditions utilized in production bases. The dry stems or slices were grounded into powders and stored at –20 °C until analysis.

2.3. Sample extraction

0.2 g of *D. officinale* sample was weighed and transferred into a 15 mL centrifuge tube. Subsequently, 8 mL of extraction solution (acetonitrile/water, volume ratio of 3:1) was added into the tube, and the mixture was vortexed for 5 min, followed by sonication for 30 min at room temperature. After sonication, the mixture was centrifuged at 4000 g for 5 min, and the supernatant was collected for the determination of flavonoid concentrations and the analysis of antioxidant activity (Fig. S1).

2.4. Determination of 17 flavonoid compounds by QuEChERS-HPLC-MS/MS

2.4.1. Sample clean-up

1.6 mL of the sample extract was transferred to a centrifuge tube which contained 50 mg of C18. The mixture was vortexed and then centrifuged at 5018 g for 5 min. Afterwards, the supernatant was collected and analyzed using HPLC-MS/MS (Fig. S1). For analysis of flavonoids with a high content level, such as naringin, rutin and schaftoside, samples were diluted by a factor of 100 using acetonitrile/water

(3:1, v/v) and then filtered through a 0.22 μm filter. On the other hand, samples were directly filtered through a 0.22 μm filter for analysis of flavonoid components with a relative low content

2.4.2. HPLC-MS/MS conditions

The flavonoid components were measured using HPLC-MS/MS (LCMS 8050, Shimadzu, Japan). Chromatographic separation was achieved on an ACQUITY UPLC HSS T3 column (2.1 mm \times 100 mm, 1.8 μm) at 40 $^{\circ}\text{C}$, and the eluent was 0.1 % formic acid in water with 2 mmol/L ammonium acetate (A) and methanol (B) at a flow rate of 0.25 mL/min. The injection volume was 2 μL . The gradient elution was performed as shown: 0–0.5 min 0–10 % B, 0.5–3 min 10 %–85 % B, 3–5 min 85 %–95 % B, 5–9 min 95 %–95 % B, 9–9.01 min 95 %–10 % B.

The mass system was equipped with an electrospray ionization (ESI) source operating in the negative mode. The nebulizer and drying gases were 99.95 % nitrogen, and their flow rates were 3.0 and 10.0 L/min, respectively. The heating gas was 99.95 % air with a flow rate of 10.0 L/min. Argon (99.99 %) was utilized as the collision gas at a pressure of 270 kPa. Other parameters were as follows: interface voltage of 4.0 kV, interface temperature of 300 $^{\circ}\text{C}$, desolvation line temperature of 250 $^{\circ}\text{C}$, heat block temperature of 400 $^{\circ}\text{C}$, and detector voltage of 1.82 kV. Data was collected using the multiple reaction monitoring (MRM) mode. The MS/MS transition for quantification and confirmation and the retention time for each target chemical were summarized in Table S3. The MRM chromatograms of 17 components were presented in Fig. S2.

2.5. Method validation and uncertainty evaluation

The method used for analysis of 17 flavonoid compounds in *D. officinale* was validated according to the guidelines presented by European Commission (2021), and the International Union of Pure and Applied Chemistry (IUPAC, 2002). The linearity, sensitivity, accuracy and precision of the method were evaluated.

The linearity was evaluated by building external calibration curves for each compound. Each calibration curve was plotted with a minimum of five suitable concentrations within the range of 1 to 200 $\mu\text{g/L}$. The sensitivity of the method was determined by the limit of detection (LOD) and the limit of quantification (LOQ), which corresponded to the concentration of each analyte at a signal-to-noise ratio of 3 and 10, respectively. The accuracy was assessed by recoveries. Mixed standards containing 17 flavonoid components were spiked at three concentration levels, namely low, medium and high (80 %, 100 % and 120 % of the original amount) into the sample, and the recovery was calculated using the formula: Recovery (%) = (total mass in spiked sample – mass in non-spiked sample)/mass spiked \times 100 (Xin et al., 2023). Both fortified and unfortified samples were treated identically. The precision of the method was evaluated by intra-day and inter-day variations.

Uncertainty of the measurement was assessed by the combination of individual uncertainty including purity of the standards, balance weighing (weighing of samples and standards), volume measuring by using a pipette (measuring of standard solutions and solvents), volumetric flask used for preparation of standard solution, and method repeatability obtained by the RSD of the measurements (ISO, 1993). The combined relative standard uncertainty (u) was calculated by Eq. (1). The expanded relative uncertainties (U) with a coverage factor of 2 ($k = 2$) was estimated by Eq. (2).

$$u = (u_{rel,p}^2 + u_{rel,w}^2 + u_{rel,m}^2 + u_{rel,v}^2 + u_{rel,rep}^2)^{0.5} \quad (1)$$

$$U = u \times 2 \quad (2)$$

$u_{rel,p}$, $u_{rel,w}$, $u_{rel,m}$, $u_{rel,v}$ and $u_{rel,rep}$ indicated the relative uncertainty of purity of standards, balance weighing, volume measuring, volumetric flask in preparation of standard solution and the method repeatability, respectively.

2.6. Total contents of flavonoid determined by $\text{NaNO}_2\text{-Al}(\text{NO}_3)_3$ colorimetry

The total contents of flavonoid in *D. officinale* (C_t) were determined according to the method of Zhishen et al. (1999) with minor modifications. 2 mL of the sample extract was first mixed with 0.3 mL of 5 % NaNO_2 (w/w). Afterwards, 0.3 mL of 10 % $\text{Al}(\text{NO}_3)_3$ (w/w) and 4 mL of 4 % NaOH (w/w) were added and the sample was then thoroughly mixed. The absorbance was measured at 510 nm. Rutin was used as a standard, and the calculation of C_t was based on rutin equivalents (mg RE/g).

2.7. Antioxidant assays

The antioxidant activity of *D. officinale* was evaluated by using the DPPH radical scavenging capacity, ABTS assay and FRAP total antioxidant capacity according to available method (Jin et al., 2016; Zhang et al., 2017; Meng et al., 2019). Additionally, three specific methods for the determination of antioxidant activities were presented in Supplemental Materials.

2.8. Calculations and statistical analysis

To test the significance of the difference in C_t under different extraction conditions, t -test was performed using IBM SPSS Statistics 25 with a statistical significance level of 0.05. Spearman correlation was applied to determine the relationship between the antioxidant activity of 47 *D. officinale* samples and the total content of flavonoid (C_t) and the individual flavonoid concentration. Moreover, the relationship between flavonoid contents and factors including origin, cultivation method and growth years was explored by the Multiple Factor Analysis (MFA) performed on XLSTAT 2018 (Addinsoft, USA). In MFA analysis, flavonoid contents and antioxidant activity values were standardized by Z-score. Considering that *D. officinale* slices were obtained by cutting the dry stem into slices, and the mechanic cutting would not cause variations in flavonoid contents, the factor 'processing' was sorted as dry stem and Fengdou, and slices were merged with dry stem.

3. Results and discussion

3.1. Method development for determination of 17 flavonoid compounds using QuEChERS-HPLC-MS/MS

3.1.1. Optimization of HPLC-MS/MS conditions

To achieve a satisfactory resolution, sensitivity and short sample running time, various mobile phases were tested. The pKa for flavonoid compounds is from 5.7 to 7.52, and LogP values vary from –3.06 to 3.19 (Table S4). Considering the acid-base property and wide polarity of flavonoid compounds, four mobile phases were examined. These were A (water – methanol), B (0.1 % formic acid – methanol), C (2 mmol/L ammonium acetate containing 0.1 % formic acid – acetonitrile) and D (2 mmol/L ammonium acetate containing 0.1 % formic acid water – methanol). A mixed standard solution of 17 flavonoid components with a concentration of 0.1 mg/L was injected, and the peak shape and peak area under the four mobile phases were compared. The peak shapes were comparable under all four mobile phases, appearing sharp and symmetrical. The peak areas of the 17 flavonoids under the four mobile phase conditions were shown in Fig. 1. Under each mobile phase, the intensity of chrysoeriol was the highest, which was nearly 64 times that of kaempferol. Meanwhile, the peak area of kaempferol was the highest under the mobile phase D. Herein, mobile phase D (2 mmol/L ammonium acetate containing 0.1 % formic acid water – methanol) was chosen for the analysis of flavonoid compounds. Furthermore, optimization of MS parameters is another essential step for obtaining the highest sensitivity. The precursor ion was determined in full scan with an m/z range of 100–1000 followed by the optimization of MRM

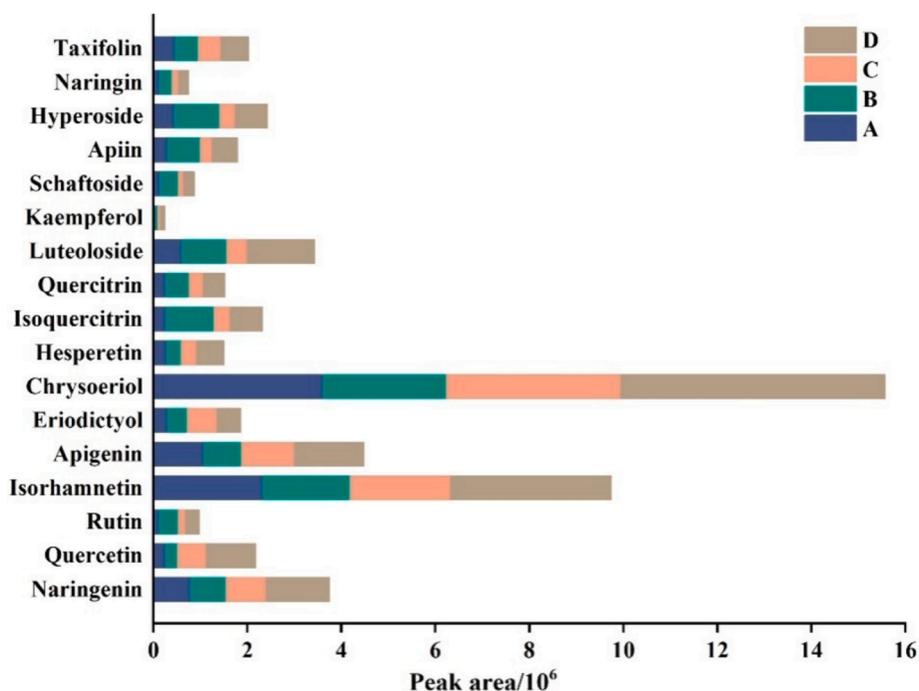


Fig. 1. Peak areas of flavonoid compounds under the four mobile phases. A (water – methanol), B (0.1 % formic acid – methanol), C (2 mmol/L ammonium acetate containing 0.1 % formic acid – acetonitrile) and D (2 mmol/L ammonium acetate containing 0.1 % formic acid water – methanol).

transitions and the corresponding collision energy (CE). The optimized MRM transitions and CE for 17 flavonoid components were listed in Table S3.

3.1.2. Optimization of extraction conditions

Efficient and reproducible sample extraction is critical to the chemical analysis. Currently, flavonoids in herbs and foods are extracted using methods including hot water bath, Soxhlet extraction, ultrasonic assisted-extraction and microwave-assisted extraction (Søltøft et al., 2009; Wong et al., 2017; Moldovan et al., 2019; Manganet al., 2020). Ultrasound assisted-extraction shows advantages of simple operation, low cost and high yield since ultrasonic treatment could induce cavitation effects and destroy cell walls efficiently to promote solute transfer from the solid phase to the liquid phase (Cui et al., 2022; Chakraborty et al., 2020). Herein, the ultrasound-assisted extraction technology was selected to extract flavonoids from *D. officinale*. Further, the extraction procedure was optimized by investigating the influence of various factors such as types of organic solvent, water content, solid–liquid ratio and extraction time. The results were shown in Fig. 2.

The extraction efficiencies of four organic solvents (methanol, acetonitrile, acetone and ethanol) were compared under constant parameters (water content of 25 %, solid–liquid ratio of 1:40 (g/mL) and an extraction time of 30 min). Acetone resulted in the highest C_t of 3.71 mg/g, followed by acetonitrile with a C_t of 3.60 mg/g (Fig. 2a). It was indicated that there was no significant differences in C_t values under these two extraction solvents (t -test, $p > 0.05$). Considering the volatility and toxicity of acetone, acetonitrile was selected as the extraction solvent. Subsequently, the effect of water content in the extraction solvent on extraction efficiency was investigated when acetonitrile was used as extraction solvent with a solid–liquid ratio of 1:40 (g/mL) and an extraction time of 30 min. The highest C_t of 3.77 mg/g was achieved when the water content was 25 % (Fig. 2b). Therefore, it was concluded that the optimal extractant was consisted of acetonitrile/H₂O (75/25, v/v). Extraction time was critical in achieving optimal ultrasound-assisted extraction efficiency. The C_t was measured at intervals between 15 and 75 min while keeping other parameters constant (acetonitrile/H₂O of 75/25, solid–liquid ratio of 1:40 (g/mL)). The flavonoid content

increased initially and then stabilized after 30 min (Fig. 2c). It was appropriate to select 30 min as ultrasonic time. Finally, the effect of solid–liquid ratio on the C_t was assessed using the optimal conditions obtained above. When the solid–liquid ratio was 1:10 (g/mL), the sample matrix was not completely immersed by the extraction solvent, resulting in incomplete extraction and low flavonoid extraction efficiency. As shown in Fig. 2d, there was no significant difference (t -test, $p > 0.05$) between C_t values at ratios of 1:40 (g/mL) and 1:50 (g/mL). Therefore, a solid–liquid-ratio of 1:40 (g/mL) was selected for sample extraction to reduce solvent usage.

3.1.3. Optimization of the QuEChERS method

D. officinale is a complex matrix containing polysaccharides, proteins and pigments (Chen et al. 2021), and the purification of the extract is vital for the analysis of flavonoid components. Herein, we developed a QuEChERS method for analyzing flavonoid compounds in *D. officinale*. The effectiveness of the sorbent depends on selective adsorption capacity, physicochemical properties, for instance functional groups and polarity, and the characteristics of the sample extract, such as pH and ionic strength (Dąbrowski et al. 2005). Five types of adsorbents (PSA, C18, g-MWCNTs, MWCNTs-OH, MWCNTs-COOH) were tested and the dosage was optimized. The amount of sorbent tested in this study was determined based on the optimized dosage reported in previous studies (Li et al., 2020; Xu et al., 2021; Garcia-Vara et al., 2022; Shinet et al., 2022). The effect of sorbents on purification efficiency was evaluated by examining the variations in flavonoid contents and color of extracts before and after purification. The concentration ratio of flavonoid component in the extract before and after purification (R_f) was presented in Table 1, implying the adsorption capacity of the adsorbent. It was indicated that PSA and C18 had a minimal adsorption capacity for flavonoids, while g-MWCNTs showed adsorption of isorhamine, apigenin, kaempferol and apiin, with R_f values ranging from 56 % to 68 %. MWCNTs-COOH and MWCNTs-OH could adsorb 76.5 % and 64.7 % of flavonoid components, respectively, showing relative strong adsorption of flavonoids in comparison to g-MWCNTs. This may be explained by that flavonoid components have a planar structure, and that the highly ordered graphite structure of g-MWCNT reduces the interaction between

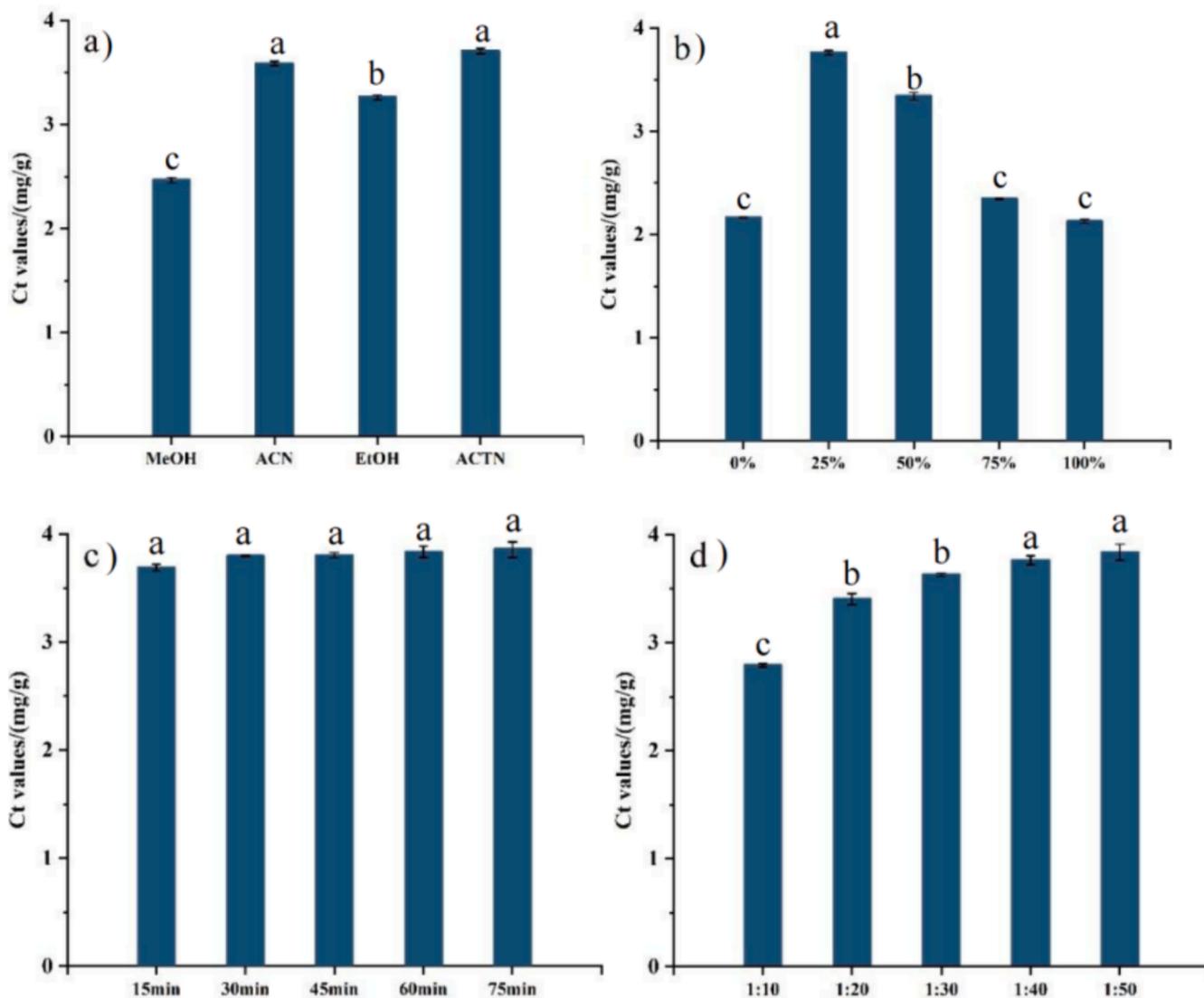


Fig. 2. Effects of extraction conditions on total content of flavonoid (C_t): (a) extraction solvents: MeOH (methanol), ACN (acetonitrile), ACTN (acetone) and EtOH (ethanol), (b) water content, (c) extraction time, and (d) solid-liquid ratio. The different letters in each subfigure indicated the significant difference among C_t values under various extraction conditions ($p < 0.05$).

Table 1

The concentration ratio of flavonoid components in the extract before and after purification (R_f, %).

Flavonoids	PSA			C18			g-MWCNTs		MWCNTs-COOH		MWCNTs-OH	
	75 mg	50 mg	25 mg	75 mg	50 mg	25 mg	5 mg	2.5 mg	5 mg	2.5 mg	5 mg	2.5 mg
Naringenin	106	83	81	88	81	91	81	92	72	79	79	93
Quercetin	91	83	87	91	74	86	74	82	52	61	51	58
Rutin	77	78	80	93	83	89	81	81	14	39	31	63
Isorhamnetin	99	90	82	88	79	77	60	60	26	39	27	47
Apigenin	107	99	89	96	85	79	62	69	25	48	30	53
Eriodictyol	97	99	94	92	100	81	90	86	67	79	91	106
Chrysoeriol	78	85	85	85	86	80	73	86	37	59	37	54
Hesperetin	101	109	101	102	114	106	103	110	101	108	98	99
Isoquercitrin	84	78	83	87	87	73	73	73	20	41	41	64
Quercitrin	81	78	74	93	84	73	87	70	20	36	38	58
Luteoloside	85	79	103	89	98	78	77	85	10	37	70	41
Kaempferol	76	75	82	76	90	80	56	39	50	31	62	45
Schaftoside	73	80	77	96	88	93	83	91	58	74	73	81
Apiin	89	96	96	97	97	99	68	89	19	44	32	58
Hyperoside	75	82	89	91	92	87	75	79	25	44	34	53
Naringin	79	92	116	102	95	108	117	103	70	88	81	84
Taxifolin	85	93	93	98	101	100	95	102	82	102	88	87

adsorbents and flavonoid components (Zhu et al., 2019; Li et al., 2020). Moreover, PSA removed less pigment than C18 indicated by the negligible variation in the color of sample extract treated by PSA (Fig. S3). Correspondingly, C18 was chosen as the purification sorbent. When considering the dosage of C18, the sample extract had comparable light yellow in color when C18 dosage was changed from 50 to 75 mg. Consequently, 50 mg of C18 was applied in the sample clean-up.

3.2. Method validation and comparison

The QuEChERS-HPLC-MS/MS method was validated to verify that its performance was qualified for the routine analysis of flavonoid components in *D. officinale*. Performance parameters including linearity, sensitivity, accuracy and precision were assessed.

All flavonoids showed a good linearity in the range of 1–200 µg/L, and the correlation coefficient was greater than 0.99 (Table 2). The LOD of 17 flavonoid components ranged from 2.1 to 49.55 µg/kg, and the LOQ ranged from 7.12 to 165.2 µg/kg (Table 2). The recoveries were examined at three levels, providing recoveries of 73 % to 112 %. The intra-day precision and inter-day precision were varied from 1.9 % to 13.3 % and 3.7 % to 15.1 %, respectively. Therefore, the validation results met the criteria of SANTE/11312/2021 (European Commission, 2021), demonstrating that the QuEChERS-HPLC-MS/MS method can provide good linearity, sensitivity, accuracy as well as precision for simultaneous analysis of 17 flavonoid components in *D. officinale*. Additionally, the relative uncertainties of 17 flavonoid components were in the range of 2.4 % to 15.4 % and the expanded relative uncertainties were in the range of 4.8 % to 30.8 % (Table S5).

The quantitative analysis of 17 flavonoid components in this work applied QuEChERS method in sample preparation to reduce the interference in sample extracts. The study on the quantitative analysis of flavonoid components in *D. officinale* was relative rare and only one relevant publication was available. The method developed in this work was compared with those reported on *D. catenatum* flowers, *D. chrysotoxum* flowers and *D. officinale*, and sample extraction and instrumental analysis was summarized in Table S6. The method in this work cost less amount of solvent and less sample preparation time, and afforded higher sensitivity when compared with Zhang et al. (2019) and Hu et al. (2023). Meanwhile, the method in this work showed comparable sensitivity with that of Lv et al. (2017).

3.3. Total content of flavonoid in *D. officinale*

The total content of flavonoid (C_t) ranged from 1.57 to 9.36 mg/g, with an average value of 4.21 mg/g. The highest C_t of 9.36 mg/g was

observed for sample 4 collected from Panan, Zhejiang Province, while the lowest C_t of 1.57 mg/g was measured in sample 44 taken from Taizhou, Zhejiang Province. Samples (1–4) were obtained from the same production base located in Jinhua, Zhejiang Province, being grown under imitative wild cultivation for 2, 3, 4 and 5 years, respectively. The C_t value of samples 1–4 indicated that the total content of flavonoid was enhanced with the increase of growth year (Fig. 3). The finding was similar with the study of Xin et al. (2015) who observed that flavonoid contents in *Astragali Radix* was increased with the extended growth time.

3.4. Contents of 17 flavonoid components

Flavonoids belong to a large group of secondary metabolites in *D. officinal*. The basic skeletons of flavonoids are apigenin, vitexin, quercetin and kaempferol. In this study, we employed the validated method to determine the content of 17 flavonoid components with available standards in *D. officinal* samples from Zhejiang, Yunnan, Anhui and Guangxi Provinces. The total concentration of 17 flavonoid components is denoted as C_{17} , ranging from 33.09 to 655.44 mg/kg. It was observed that naringenin, rutin and schaftoside were the three predominant flavonoid components in *D. officinal* (Fig. 4a), accounting for more than 80 % of the C_{17} . The concentration of naringenin, rutin and schaftoside in *D. officinal* were 5.19–57.18, 2.01–563.17 and 4.43–67.73 mg/kg, respectively. The highest concentration of naringenin, rutin and schaftoside were observed in samples 4, 43 and 35, respectively. The cultivation methods for these three samples were imitative wild cultivation, while their origins were Jinhua, Quzhou and Taizhou, Zhejiang Province, respectively. The concentration of the remaining 14 flavonoid components in *D. officinal* was relatively low, accounting for only 1.05 %–16.83 % of the C_{17} measured. The variation in individual flavonoid concentrations with extended growth years was examined in samples 1–4, which were grown under the same cultivation method yet for different years. It was noted that the variation of flavonoid components with growth years were compound-dependent. For example, the contents of naringenin, isorhamnetin, eriodictyol, kaempferol and taxifolin increased with the extension of growth year, while the contents of chrysoeriol, quercetin, and apiin remained relatively stable for 2–4 years before reaching their highest levels in the fifth year. This might be due to the different functions of flavonoids.

3.5. Antioxidant capacity of *D. officinale*

D. officinale sample extracts were screened for antioxidant capacity by using DPPH, ABTS, and FRAP assays (Zhao et al., 2021). DPPH radical

Table 2
Linearity, sensitivity, accuracy and precision of the method.

No.	Flavonoids	Calibration curve (1–200 µg/L)	r^2	LOD (µg/kg)	LOQ (µg/kg)	Recovery (%)			Intra-day RSD (%)	Inter-day RSD (%)
						Low	Medium	High		
1	Naringenin	$y = 10770x + 2940.5$	0.9997	4.3	14.3	83	78	97	3.0	9.9
2	Quercetin	$y = 3749.5x - 9400.3$	0.9979	31.4	104.8	85	83	78	2.3	6.7
3	Rutin	$y = 1851.1x - 2159$	0.9997	4.6	15.2	83	80	89	6.9	13.8
4	Isorhamnetin	$y = 19393x - 10,138$	0.9962	5.3	17.8	103	112	106	1.9	3.7
5	Apigenin	$y = 11375x - 2195.9$	0.9995	2.3	7.8	73	84	78	6.0	12.2
6	Eriodictyol	$y = 4030.4x - 7641.3$	0.9992	49.5	165.2	107	107	100	7.1	8.1
7	Chrysoeriol	$y = 40809x - 9176.6$	1.000	6.2	20.6	87	85	80	3.1	7.8
8	Hesperetin	$y = 5106.5x - 669.98$	0.9999	7.4	24.5	98	95	88	2.3	6.4
9	Isoquercitrin	$y = 4800.4x - 4315$	0.9997	4.1	13.5	92	91	85	8.0	9.1
10	Quercitrin	$y = 2994.4x - 3977$	0.9997	2.1	7.1	95	97	98	13.3	9.9
11	Luteoloside	$y = 9930.4x - 5001.7$	0.9999	11.3	37.8	86	82	86	6.5	13.2
12	Kaempferol	$y = 706.74x - 1781.6$	0.9974	11.2	37.2	91	90	94	6.4	8.5
13	Schaftoside	$y = 1659x - 805.88$	0.9998	15.0	50.1	87	88	96	11.2	12.5
14	Apiin	$y = 4543.7x - 5884$	0.9996	16.7	55.6	101	95	95	5.4	10.4
15	Hyperoside	$y = 4222.4x - 5855.4$	0.9995	6.1	20.2	111	100	106	5.5	7.4
16	Naringin	$y = 1680.3x + 967.08$	0.9999	17.2	58.0	92	93	91	3.5	15.1
17	Taxifolin	$y = 3314.4x - 4672.6$	0.9996	48.8	162.4	102	111	101	11.8	7.8

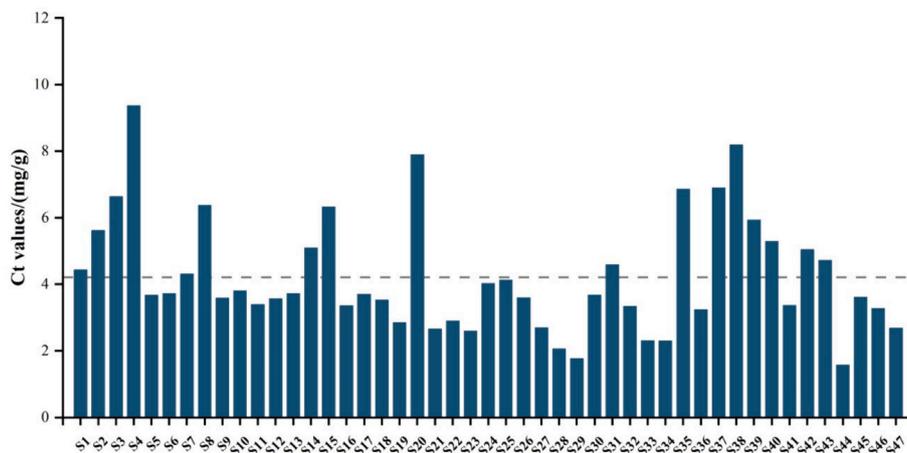


Fig. 3. Total contents of flavonoid (C_t) in 47 of *D. officinale* samples.

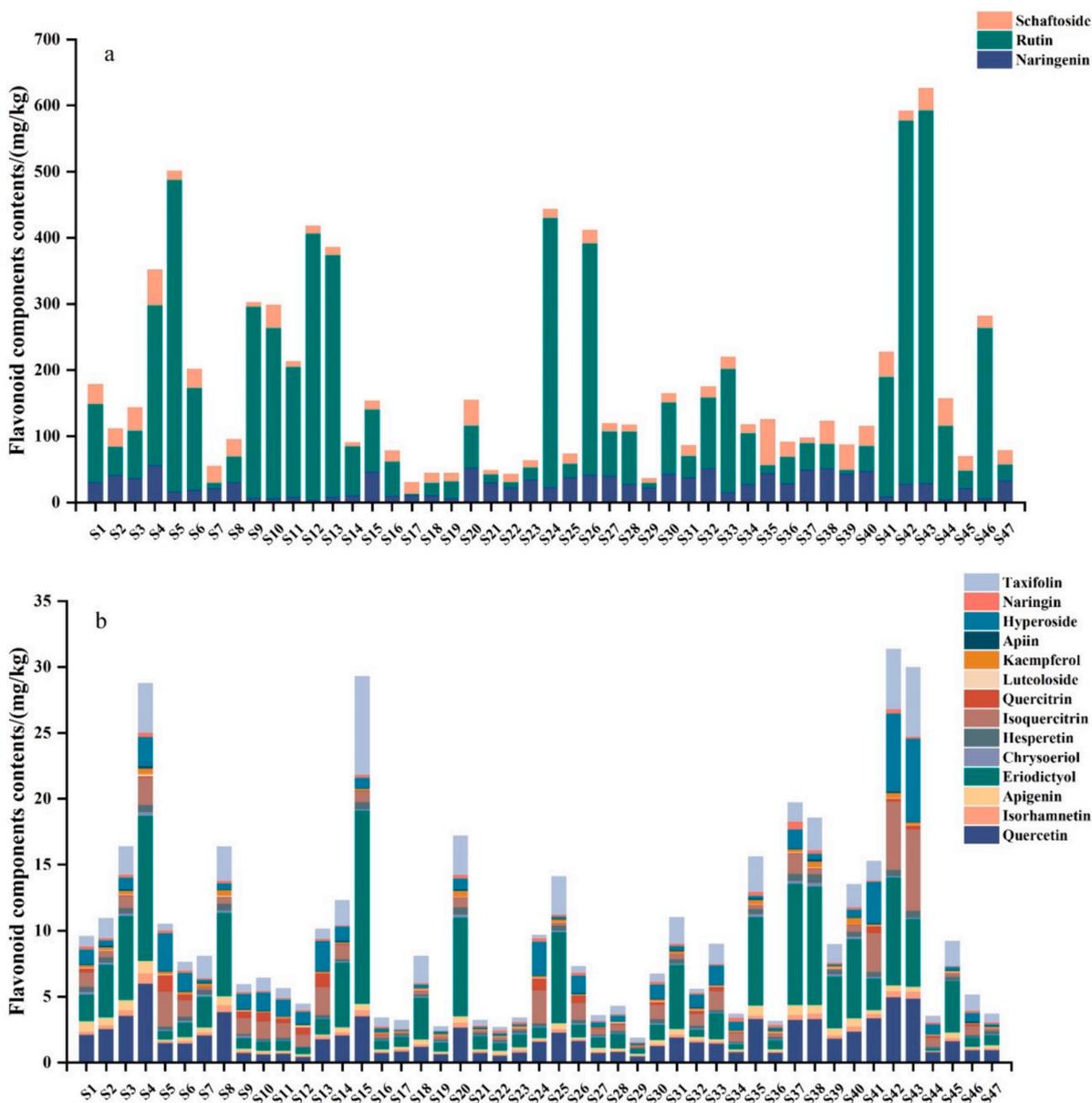


Fig. 4. 17 flavonoid components in 47 of *D. officinale* samples. (a) Contents of naringenin, rutin and schaftoside, and (b) Contents of the remaining 14 flavonoid components.

scavenging capacity in *D. officinale* sample extracts ranged from 35.62 % to 89.64 %, a 2.5-fold variations (Table S7). ABTS free radical scavenging rate varied from 16.77 % to 96.02 %, a 5.7-fold differences (Table S7). FRAP values ranged from 277.51 to 1523.41, a 5.5-fold

variations (Table S7). Sample 29 from Mangshi, Yunnan Province showed the lowest antioxidant capacity indicated by three antioxidant capacity assays, while sample 15 from Lishui, Zhejiang Province had the highest DPPH radical scavenging ability. Sample 4 from Jinhua,

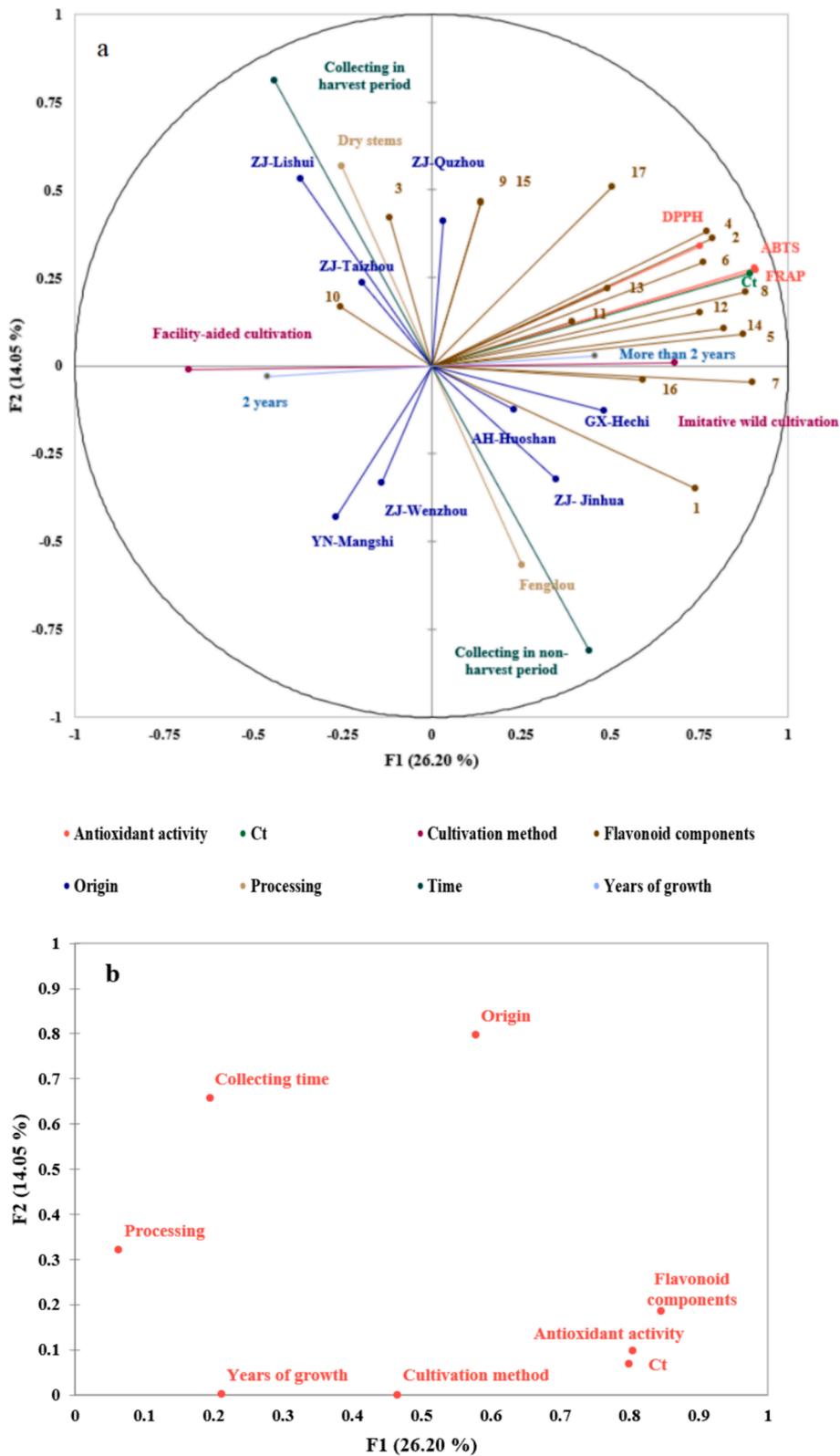


Fig. 5. The results of MFA analysis (1-Naringenin, 2-Quercetin, 3-Rutin, 4-Isorhamnetin, 5-Apigenin, 6-Eriodictyol, 7-Chrysoeriol, 8-Hesperetin, 9-Isoquercitrin, 10-Quercitrin, 11-Luteoloside, 12-Kaempferol, 13-Schaftoside, 14-Apiin, 15-Hyperoside, 16-Naringin and 17-Taxifolin). (a) Correlation map of all variables, (b) coordinates of the eight variable classes, (c) observation scores plot of MFA (cultivation method) and (d) observation scores plot of MFA (origin).

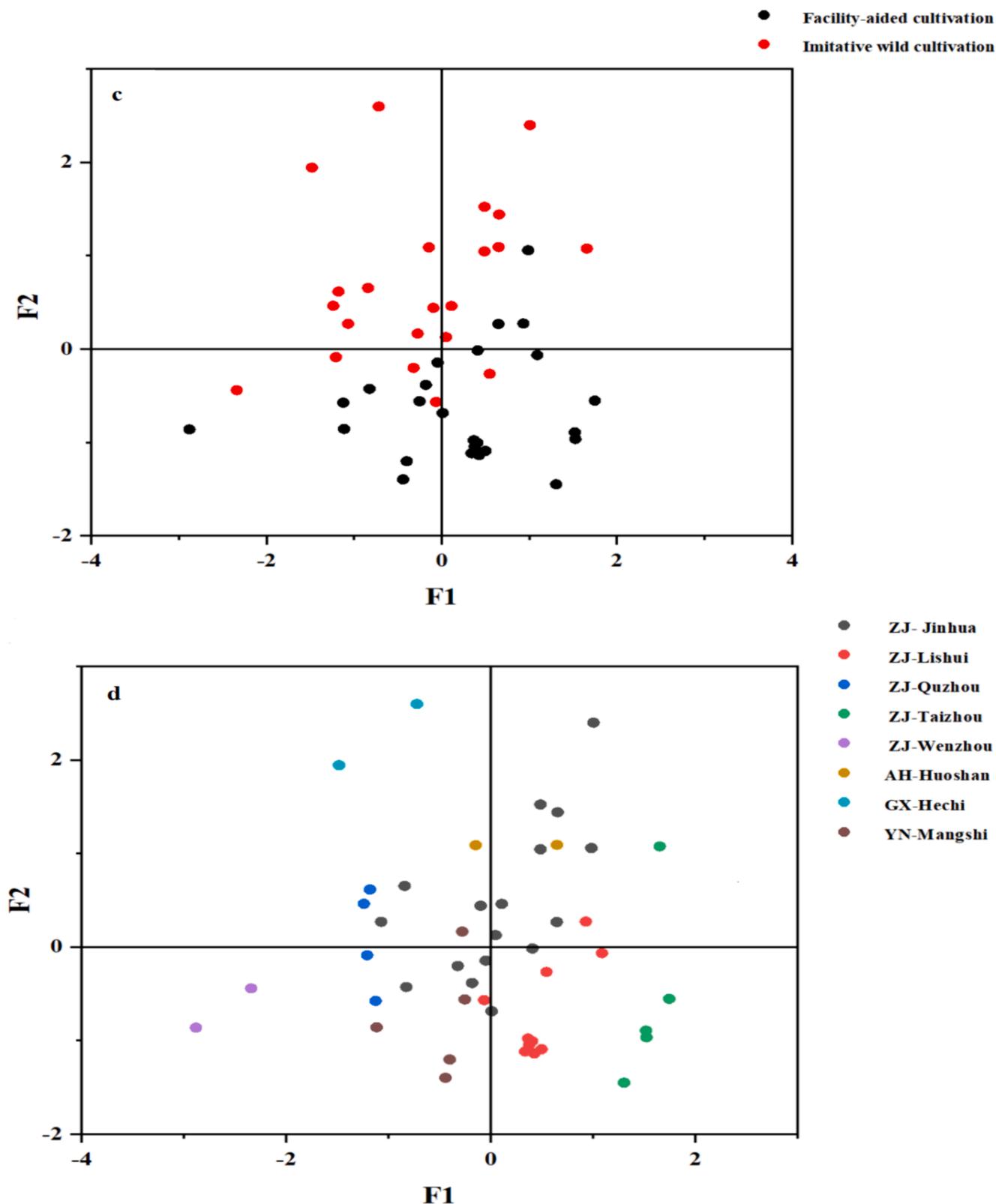


Fig. 5. (continued).

Zhejiang Province exhibited the highest ABTS and FRAP antioxidant activities. Correlation analysis was performed to investigate the relationship between the flavonoid concentrations and antioxidant activities of *D. officinale* sample extracts (Table S8). The correlation coefficients between C_t and DPPH radical scavenge, ABTS radical scavenge and total antioxidant capacity of FRAP exceeded 0.7, highlighting the important

role of flavonoids in the antioxidant ability of *D. officinale*. As regard to flavonoid compounds, 12 out of 17 compounds, including naringenin, quercetin, apigenin and isorhamnetin, were significantly positively correlated with the antioxidant activity determined by the three assays. The results were comparable with Huang et al. (2014), who found the correlation between the content of flavonoids and the antioxidant

activity of *D. officinale* extracts. Furthermore, quercetin, one of most well-known flavonoids, has observed to be a powerful scavenger of reactive oxygen species (Wang et al., 2016).

3.6. Relationship among flavonoid components, antioxidant activity and influencing factors

Flavonoid contents in *D. officinale* may be influenced by various factors such as origin, cultivation method, growth years, collecting time and processing techniques. MFA was applied to study the relationship among the origin, processing, growth years, collection period, cultivation method, total flavonoid content (C_t), 17 flavonoid component contents and antioxidant activity of *D. officinale* (Fig. 5). The first two factors exhibited a variability of 26.20 % and 14.05 %, respectively. 12 out of 17 flavonoid compounds, including quercetin, isorhamnetin, eriodictyol, schaftoside, luteoloside, apiin, hesperetin, taxifolin, apigenin, kaempferol, hyperoside and isoquercitrin were clustered together in the first quadrant. On the other hand, rutin, naringin, and quercetin were separated from these 12 flavonoid compounds. The C_t and antioxidant activity were found to be closely related to the 12 flavonoid components since they were clustered in the near proximity, and it suggested that those 12 flavonoids contributed significantly to the antioxidant activity. This observation was consistent with the results obtained from correlation analysis. Eight different origins of *D. officinale* were distributed across four quadrants. Contents of 12 flavonoid components were relatively high in *D. officinale* sampled from Quzhou, Guangxi and Anhui compared to samples from Yunnan, Wenzhou and Taizhou, and it was consistent with the fact that samples obtained from Quzhou, Guangxi and Anhui were found to be closely associated with the 12 flavonoid components. A positive correlation was found between the *D. officinale* collected from Taizhou and quercetin. Accordingly, the contents of 12 flavonoid components in samples grown under imitative wild cultivation were observed to be relatively high compared to those cultivated in green-house. Moreover, long-growth-year (more than 2 years) was favored for accumulating these 12 flavonoid components. Fig. 5b plotted the coordination of all classes and indicated that the origin and cultivation methods contributed more to the flavonoid content and antioxidant activity because they have a more proximate relationship. By differentiating the color of sample dot according to cultivation method, *D. officinale* samples cultivated under facility-aided and imitative wild methods were mainly scattered in two separate areas on MFA score plot (Fig. 5c). Nevertheless, when the colour of sample dots was sorted according to the origin, samples from Jinhua, Quzhou and Lishui had large overlapping, suggesting that multiple factors other than the origin account for the accumulation of flavonoid in *D. officinale*.

4. Conclusion

A reliable and sensitive QuEChERS-HPLC-MS/MS method was established to simultaneously determine 17 flavonoid compounds in *D. officinale*. The sample was ultrasonic-extracted with 75 % acetonitrile followed by purification using C18. The analytical performance was satisfactory and was successfully applied to 47 of *D. officinale* samples. Naringenin, rutin and schaftoside were the three predominant components in *D. officinale*, accounting for over 80 % of the total concentrations of 17 flavonoids. MFA analysis showed that the origin and cultivation method had a great influence on the flavonoid content in *D. officinale*. Correlation analysis demonstrated a positive correlation between flavonoid contents and antioxidant activity. This study provided a simple and efficient method for the measurement of 17 flavonoid components in *D. officinale*, which can aid in the function research of *D. officinale*. This work can provide guidelines for the cultivation of *D. officinale*, thus helping to further utilize and research flavonoids in *D. officinale*. In future, if more flavonoid standards are available, the method could be validated for these flavonoids and made minor modifications such as change the amount of adsorbent if necessary.

CRediT authorship contribution statement

Fengting Sun: Writing – original draft. **Zhenlan Xu:** Conceptualization, Writing – review & editing. **Xiaoyan Xu:** Methodology. **Yan Gao:** Formal analysis. **Zuoyi Zhu:** Formal analysis. **Xinyu Han:** Methodology. **Chunrong Zhang:** Validation. **Tao Tang:** Methodology. **Qiang Wang:** Conceptualization. **Qing Sheng:** Project administration. **Xueping Zhao:** Project administration.

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 material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.arabjc.2024.105911>.

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