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Identification and determination of the major constituents in Deng's herbal tea granules by rapid resolution liquid chromatography coupled with mass spectrometry

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ABSTRACT

Deng's herbal tea (DHT), a famous traditional Chinese herbal tea consisting of six traditional Chinese medicines (*Honeysuckle*, *Chrysanthemum*, *Rhizoma imperatae*, *Folium mori*, *dandelion* and *liquorice*), is widely used in China for its health benefits. In this paper, a rapid resolution liquid chromatography coupled with mass spectrometry (RRLC–MS) method was developed for the identification and determination of the major constituents in DHT granules. A good RRLC separation was achieved using an Agilent Poroshell 120 SB-C_{18} column and gradient elution (0.5% formic acid in water/acetonitrile) within 30 min. Twenty-eight compounds were identified or tentatively characterized based on their exact molecular weights and fragmentation patterns. Fifteen major bioactive constituents of those 28 compounds were chosen as the benchmark substances. Their quantitative analyses were performed by a triple quadrupole tandem mass spectrometer (MS/MS) operating in multiple-reaction monitoring mode, and a full quantitative analysis of the 15 major constituents was performed by our developed RRLC–MS/MS method in only 10 min. Of the 16 DHT granule samples tested, the quality of the results was stable, which confirms that the developed method was efficient and robust for the quality control of DHT granules.

1. Introduction

Traditional Chinese herbal tea (TCHT) is a medicinal beverage made from traditional Chinese medicine (TCM) and has been widely used in China for its health benefits [1–3]. In 2006, TCHT was approved as an intangible cultural heritage by the Chinese government. Deng's herbal tea (DHT), a famous TCHT consisting of six TCMs, i.e., *Honeysuckle*, *Chrysanthemum*, *Rhizoma imperatae*, *Folium mori*, *dandelion* and *liquorice*, is widely used for the prevention and treatment of the flu, fever, sore throat and other diseases. Caffeoylquinic acids (CQAs), flavonoids and triterpenoids are considered to be the main active compounds in DHT formula. CQAs show physiological activities, such as anti-inflammatory, anti-microbial, anti-oxidative and other protective effects [4–9]. Flavonoids possess anti-bacterial, anti-fungal, anti-viral and anti-cancer activities [10–13]. Triterpenoids have been widely studied because of their various pharmacological activities [14–19]. These compounds are also often selected as the markers for quality evaluations of TCM [4].

A rapid, efficient analysis method for a comprehensive evaluation is necessary to ensure the quality of TCHT. In the past several years, quality evaluations of TCM have been performed by using thin layer chromatography [20], gas chromatography [21], capillary electrophoresis [22] and high performance liquid chromatography (HPLC) [23,24]. In recent decades, HPLC coupled with mass spectrometry (MS) with excellent sensitivity and specificity has been proven to be a powerful approach to simultaneously characterize and determine the different constituents in multi-herbals and in their preparations [25,26]. Recently, rapid resolution liquid chromatography (RRLC) coupled with evaporative light scattering detection [27] and MS [28] has also been applied to analyze TCMs. Using highly linear velocity columns packed with 1.8-μm porous particles or 2.7-μm porous shell particles (which consist of 1.7-μm solid silica cores with 0.5-μm porous outer layers), the separation efficiency of RRLC is remarkably improved comparing with that of a conventional HPLC system. The time required to perform a full analysis run by RRLC is about one-fourth of that by HPLC [27,28]. Furthermore, when time-of-flight mass spectrometry (TOF-MS) and ion-trap mass spectrometry (IT-MS) are coupled

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to an RRLC system, straightforward identifications of constituents can be obtained based on their exact molecular weights and MS fragment patterns [28].

In this study, we apply an RRLC–MS method for the identification and determination of the major constituents in DHT granules. Twenty-eight compounds were identified or tentatively characterized by RRLC–TOF–MS and RRLC–IT–MS. Fifteen bioactive constituents of those 28 compounds were quantitative analyzed by RRLC coupled with a triple quadrupole tandem mass spectrometer (RRLC–MS/MS). Sixteen batches of samples were analyzed and the results were expected to provide comprehensive information for the quality control of DHT granules.

2. Materials and methods

2.1. Materials and reagents

Acetonitrile and methanol of HPLC grade were purchased from Burdick & Jackson (Muskegon, MI, USA). Water for RRLC analysis was purified by a Milli-Q water-purification system (Milford, MA, USA). Formic acid was analytical grade and purchased from Guangzhou Chemical Reagent Factory (Guangzhou, China).

Fifteen reference substances, 3-*O*-CQA, 4-*O*-CQA, 5-*O*-CQA, 3,4-di-*O*-CQA, 3,5-di-*O*-CQA, 4,5-di-*O*-CQA, linarin, liquiritin, rutin, luteolin-7-*O*-*β*-D-glucoside, luteolin, dipsacoside B, glycyrrhizic acid, macranthoidin A and macranthoidin B, were purchased from Chengdu Push Bio-Technology Co., Ltd. (Sichuan, China). Structures (Fig. 1) were elucidated based on their spectral analyses (MS, ^{1}H NMR, ^{13}C NMR, ^{1}H–^{1}H COSY, HMBC and HMQC), and their purities were found by HPLC analysis to be more than 98%.

Sixteen batches of DHT granules were purchased from different drugstores in Guangzhou; all the batches were produced by Guangzhou Yanghe Medicine-Technology Co., Ltd. (Guangzhou, China). The batch number for each sample was D10909, D11011, D11013, D11014, D11015, D11016, D11017, D11101, D21006, D21007, D21008, D21009, D21010, D21011, D21101 and D21102. Sample D21009 was used for our method development studies.

2.2. Preparation of reference solutions

Stock solutions of the 15 reference substances for quantitative determination were prepared by dissolving 5.0 mg (±0.1 mg) of each reference substance in 5.0 mL of methanol. A mixed stock solution of 50 mg/L was prepared by mixing 2.5 mL of each stock solution and diluting the mixture to 50 mL with methanol. To prepare our working solutions with different concentrations, portions of the mixed stock solution were diluted with appropriate amounts of methanol. All solutions were stored in a refrigerator at 4 °C, and they were warmed to room temperature prior to use.

2.3. Preparation of sample solutions

Five grams of a DHT granule sample was extracted with 50 mL of methanol–water (9:1, v/v) in a flask and sonicated for 15 min followed by filtration. The extraction procedure was repeated twice more, and the extracts were combined. The combined extracts were evaporated under reduced pressure at 50 °C to approximately 10 mL, transferred to a 25-mL volumetric flask, brought up to volume with methanol, and filtered through a nylon membrane filter (0.2 μm, Phenomenex, USA) for RRLC–IT–MS and RRLC–TOF–MS analysis. For RRLC–MS/MS analysis, 1.0 mL of the obtained extracts was diluted to 10.0 mL with methanol.

2.4. Instruments

Chromatographic separations were performed on an Agilent 1200 Series RRLC system (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump, microvacuum degasser, high-performance autosampler, column compartment and diode-array detector (DAD). The samples were separated on an Agilent Poroshell 120 SB-C_{18} column (100 mm × 2.1 mm i.d., 2.7 μm) at a temperature of 25 °C and using a water–formic acid (100:0.5, v/v) (solvent A) and acetonitrile (solvent B) mobile phases at a flow rate of 0.5 mL/min. To identify the major constituents in the DHT granules, a linear solvent gradient was used: 0–1 min, 5% B; 1–15 min, 5–20% B; 15–25 min, 20–45% B; 25–30 min, 45–95% B; and 3-min post-run, 5% B. Next, to determine the contents of the 15 major constituents in the DHT granules, a separate linear solvent gradient was used: 0–1 min, 10% B; 1–8 min, 10–45% B; 8–10 min, 45–95% B; and 3-min post-run, 5% B. For each system used, the DAD was set to monitor absorbance at 254 nm, and each online spectrum was recorded in the range of 190–400 nm.

For the identification of the major constituents in the DHT granules, the RRLC system was coupled to an Agilent 6210 TOF-MS (Agilent Technologies, USA) and an Agilent Trap XCT IT-MS (Agilent Technologies, USA), respectively. However, for quantitative analysis of the 15 major constituents in the DHT granules, the RRLC was coupled to an Agilent 6410 B triple quadrupole mass spectrometer (Agilent Technologies, USA). An electrospray ion source was used for all of the three mass spectrometers. Additionally, the capillary voltage was set at 3500 V, the drying-gas temperature was 350 °C, the flow rate was 10.0 L/min, and the nebulizer pressure was 50 psi. The RRLC–IT–MS spectra were scanned from a mass-to-charge ratio (*m/z*) of 50–1500 in Auto-MS^n mode, and the positive- and negative-ion MS^1 and MS^2 data were also acquired, respectively. The RRLC–TOF–MS spectra were recorded over a mass range of *m/z* 50–1500, in both positive- and negative-ion modes, respectively. A reference solution was used to account for system bias during the full RRLC–TOF–MS analysis procedure. Ions with *m/z* 118.0863 and 922.0098 in the positive-ion mode and *m/z* 112.9856 and 1033.9881 in the negative-ion mode were selected to help calibrate the mass readings of the system, and the mass accuracies of the calibrated ions were within 5 ppm. The RRLC–MS/MS system operated in multiple-reaction monitoring (MRM) mode using negative-ion pairs for the quantitative analysis of the 15 constituents, and the optimum parameters are given in Table 1.

2.5. Validation of quantitative analysis

The prepared mixed stock solution containing the 15 reference substances was diluted to form a series of appropriate concentrations for the construction of calibration curves. Here, the working solutions of eight different concentrations were injected in triplicate to obtain the calibration curves. The LODs and LOQs for each analyte were defined by the concentrations that generated peaks with signal-to-noise values (*S*/*N*s) of 3 and 10, respectively.

Intra- and inter-day variations were evaluated to determine the precision and accuracy of the method. For the intra-day variability, the mixed working solution with a concentration of 1000 μg/L for each compound was analyzed six times in a day, whereas the inter-day variability was examined in duplicate using this sample on three consecutive days.

The recoveries of each analyte was determined using the standard addition method, in which 2.5 mg of 5-*O*-CQA, 5.0 mg of 3-*O*-CQA, 2.5 mg of 4-*O*-CQA, 0.5 mg of liquiritin, 0.2 mg of rutin, 0.5 mg of luteolin-7-*O*-*β*-D-glucoside, 1.5 mg of 4,5-di-*O*-CQA, 1.5 mg of 3,5-di-*O*-CQA, 1.5 mg of 3,4-di-*O*-CQA, 0.2 mg of luteolin, 0.5 mg of linarin, 5.0 mg of macranthoidin B, 0.5 mg of macranthoidin A, 0.5 mg of dipsacoside B and 1.5 mg of glycyrrhizic

Table 1
The optimum parameters of triple quadrupole mass spectrometry for quantitative analysis of the 15 major constituents in DHT granule.

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell time (ms)	Fragmentor (V)	Collision energy (eV)
5-*O*-CAQ	353.1	191.1[a], 179.1	50	107	12
3-*O*-CAQ	353.1	191.1[a], 179.1	50	107	12
4-*O*-CAQ	353.1	173.1[a], 191.1	50	107	8
Liquiritin	417.1	255.1[a], 135.1	50	145	12
Rutin	609.1	300.1[a], 271.1	50	139	36
Luteolin-7-*O*-*β*-D-glucoside	447.1	285.1[a]	50	150	24
4,5-di-*O*-CQA	515.1	353.1[a], 179.1	50	125	12
3,5-di-*O*-CQA	515.1	353.1[a], 179.1	50	125	12
3,4-di-*O*-CQA	515.1	353.1[a], 179.1	50	125	12
Luteolin	285.0	133.1[a], 199.1	50	150	36
Linarin	591.2	283.1[a]	50	150	8
Macranthoidin B	1397.7	1073.6[a]	50	175	36
Macranthoidin A	1235.6	911.5[a]	50	180	40
Dipsacoside B	1073.6	749.5[a], 911.5	50	180	40
Glycyrrhizic acid	821.4	351.1[a], 429.5	50	169	40

[a] Quantitative ion.

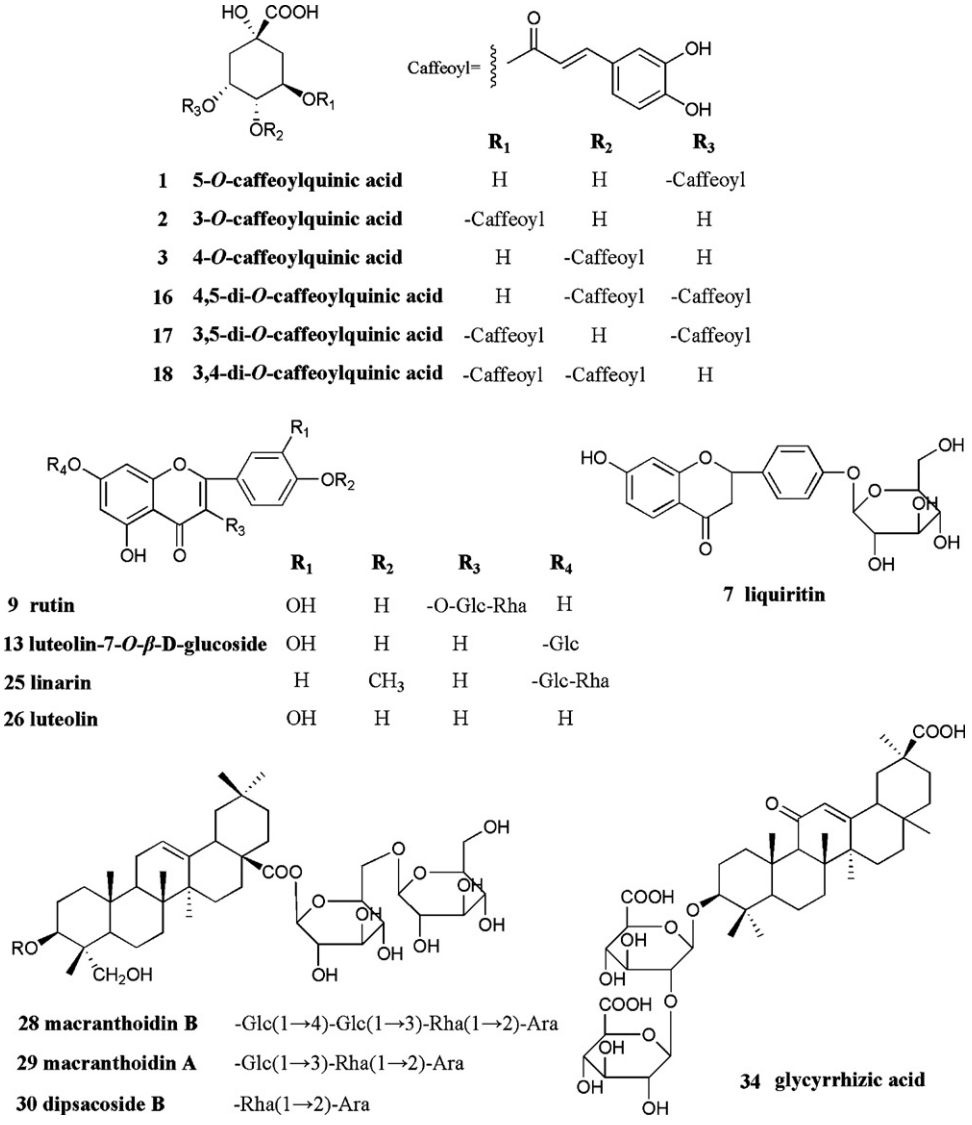

Fig. 1. Chemical structures of the 15 reference substances.

acid were spiked into a 5.0-g sample of D21009. The spiked samples were extracted and analyzed by RRLC–MS/MS using the above-described method.

3. Results and discussion

3.1. Optimization of RRLC conditions

A full separation by RRLC is necessary for the identification of as many of the constituents in the DHT granules as possible. DHT granules consist of six TCMs, and each TCM contains many constituents of similar polarities, which makes their separations quite difficult. Therefore, different elution methods using different elution solvent systems, including water–methanol, water–acetonitrile, water (containing 0.5% formic acid)–methanol and water (containing 0.5% formic acid)–acetonitrile, were investigated. The results show that a linear gradient elution with water (containing 0.5% formic acid)–acetonitrile gave the best resolution and that most of the constituents could be efficiently separated within 30 min (Fig. 2AB and C). Acetonitrile remarkably improved the separation of many of the constituents compared to methanol. Additionally the addition of formic acid had a substantial effect by increasing the retention time, depressing peak tailing of the organic acids, and strikingly improving separation efficiencies and sensitivities.

3.2. Identification of constituents in DHT

The RRLC chromatograms obtained by UV at 254 nm and the base peak chromatograms (BPC) for the positive- and negative-ion ESI–IT–MS are shown in Fig. 2A, B and C, respectively. Most of the constituents were efficiently separated using the optimized chromatography conditions. Furthermore, in the positive-ion ESI mode experiments, the protonated molecules $[M+H]^+$ were easily detected as the base peaks for nearly all of the constituents, and the ions of $[M+NH_4]^+$ were also detected as the base peaks for a few other constituents in the spectra. In the negative-ion ESI mode experiments, the deprotonated molecules $[M-H]^-$ were detected as the base peaks for most of the constituents, and the formate adducts $[M+HCOO]^-$ were also observed as the base peaks of the other constituents in the spectra. The exact molecular weight of each constituent was easily calculated according to the experimental mass of the respective pseudo-molecular ions, and the molecular formulas of those were deduced from each exact molecular weight obtained by high-resolution ESI–TOF–MS. The fragmentation information of each constituent was also obtained by ESI–IT–MS, which was quite useful for the identification of each constituent. Table 2 lists the retention times (t_R), molecular formulas, theoretical molecular weights, λ_{max}s, and ESI–TOF–MS and ESI–IT–MS ions of the 37 major peaks in the chromatograms.

Based on the exact molecular weights, MS^n data, typical UV absorption patterns and the elution orders, a total of 28 constituents from the DHT granules were identified or tentatively characterized. Among them, 6 organic acids (5-*O*-CQA (**1**), 3-*O*-CQA (**2**), 4-*O*-CQA (**3**), 4,5-di-*O*-CQA (**16**), 3,5-di-*O*-CQA (**17**) and 3,4-di-*O*-CQA (**18**)), 5 flavonoids (liquiritin (**7**), rutin (**9**), luteolin-7-*O*-*β*-D-glucoside (**13**), linarin (**25**) and luteolin (**26**)), and 4 triterpenoids (macranthoidin B (**28**) macranthoidin A (**29**), dipsacoside B (**30**) and glycyrrhizic acid (**34**)) were unambiguously identified by comparison of their t_Rs, UV spectra, and TOF–MS and IT–MS data with those of their reference substances.

The other 13 compounds were tentatively characterized as follows: isomeric di-*O*-CQA (**5**), secoxyloganin (**6**), liquiritigenin-4′-apiosyl(1-2)-glucoside (**8**), quercetin-3-*O*-*β*-D-glucoside (**12**), lonicerin (**15**), licuraside (**21**), isoliquiritin (**22**), liquiritigenin (**23**), licorice-saponin E_2 (**32**), licorice-saponin G_2 (**33**), isomeric glycyrrhizic acid (**35**), macranthoside B (**36**), and glycycoumarin (**37**) by comparing their exact molecular weights, MS^n spectra, UV absorptions and retention behaviors with those of reported compounds [4,19,29–31].

3.3. Quantitative determination of the major constituents in the DHT granules by RRLC–MS/MS

To develop a method for the quality control of the DHT granules, 15 major constituents were chosen as the marker substances, i.e., 5-*O*-CQA (**1**), 3-*O*-CQA (**2**), 4-*O*-CQA (**3**), liquiritin (**7**), rutin (**9**), luteolin-7-*O*-*β*-D-glucoside (**13**), 4,5-di-*O*-CQA (**16**), 3,5-di-*O*-CQA (**17**), 3,4-di-*O*-CQA (**18**), linarin (**25**), luteolin (**26**), macranthoidin B (**28**), macranthoidin A (**29**), dipsacoside B (**30**) and glycyrrhizic acid (**34**). These compounds are generally considered to be the bioactive constituents in TCMs. Unfortunately, the UV absorptions of macranthoidin B, macranthoidin A and dipsacoside B are quite weak; and a triple quadrupole mass spectrometer working in MRM mode was needed to provide better sensitivity, repeatability and reproducibility than those provided by TOF–MS and IT–MS. For this work, an RRLC–MS/MS method was developed for the quantitative determination of the 15 major constituents in the DHT granules.

The RRLC–MS/MS analysis was performed in both positive- and negative-ion ESI modes, respectively. However, their sensitivities towards the 15 compounds were different. Overall, the negative-ion mode provided better *S/N* ratios and was more suitable for quantitative determination. The deprotonated molecule $[M-H]^-$ was the primary peak in the spectra observed of all of the 15 compounds, and these peaks were chosen as the parent ions for the compounds. Another benefit of this system is that the triple quadrupole mass spectrometer working in MRM mode provided a better anti-interference ability for matrix than those provided by IT–MS and TOF–MS working in full scan mode. Thus, a faster chromatographic separation gradient could be used, and full analyses were completed within 10 min with a linear gradient elution of water (containing 0.5% formic acid)–acetonitrile (Fig. 3).

The linearities, ranges, regressions, LODs, LOQs, precisions, accuracies and recoveries of the method are listed in Table 3, and the high-correlation coefficient values ($r^2 > 0.9940$) obtained indicated that there were good linear correlations between the concentrations of the investigated compound and their peak areas within the test concentrations. The LODs and LOQs ranged from 1.7 to 146.5 μg/L and from 5.6 to 488.3 μg/L, respectively, while the intra- and inter-day precisions for each compound were less than 4.1 and 5.3%, respectively. Furthermore, the intra- and inter-day accuracies were in the range of 96.1–102.8% and 95.3–102.6%, respectively. The developed method showed good accuracy, with mean recoveries ranging from 94.3 to 104.8%. The results also indicated that the developed method was efficient, accurate and sensitive for quantitative determination of the major constituents in the DHT granules.

3.4. Application of analysis method to the DHT granule samples

The above RRLC–MS/MS method was applied to quantify the contents of the 15 major constituents in 16 DHT granule samples. All of the contents were calculated by the external standard method, and the mean values and SDs from the three parallel determinations of each sample are summarized in Table 4.

Of the 16 batches of DHT granule samples tested, all of the samples contained each major constituent. Furthermore, when the data for the concentrations of two samples were compared using the *t*-test, the contents in each sample were not significantly different ($P > 0.05$). One-way ANOVA comparisons of the 16 samples verified these findings ($P > 0.05$) and further indicated that there were no statistical differences among batches and that their quality was stable.

Table 2
RRLC–ESI–TOF–MS and RRLC–ESI–IT–MS identification of the constituents in DHT granule.

Peak	t_R (min)	Molecular formula	Theoretical molecular weight	λ_{max} (nm)	Experimental weight of positive ESI–TOF–MS (*m/z*)/error (ppm)	Experimental weight of negative ESI–TOF–MS (*m/z*)/error (ppm)	Positive ESI–IT–MS (*m/z*) parent ion/fragmental ions	Negative ESI–IT–MS (*m/z*) parent ion/fragmental ions	Identification	Reference
1	4.22	$C_{16}H_{18}O_9$	354.0951	326	355.1031 $[M+H]^+$/−2.09	353.0883 $[M-H]^-$/−1.40	355/163, 145	353/191, 179, 135	5-*O*-CQA [a]	[29]
2	6.42	$C_{16}H_{18}O_9$	354.0951	326	355.1030 $[M+H]^+$/−1.81	353.0885 $[M-H]^-$/−1.96	355/163, 145	353/191, 179	3-*O*-CQA[a]	[29]
3	6.86	$C_{16}H_{18}O_9$	354.0951	326	355.1031 $[M+H]^+$/−2.09	353.0886 $[M-H]^-$/−2.24	355/163, 145	353/173, 179, 191, 135	4-*O*-CQA[a]	[29]
4	8.05	$C_{18}H_{28}O_9$	388.1733	241, 314	406.2082 $[M+NH_4]^+$/−2.68	387.1667 $[M-H]^-$/−1.66	406/389, 227	387/369, 341, 207, 163	Not identify	
5	9.29	$C_{25}H_{24}O_{12}$	516.1268	326	517.1355 $[M+H]^+$/−2.80	515.1203 $[M-H]^-$/−1.55	517/499, 319, 163	515/353, 335, 173, 191	Isomeric di-*O*-CQA	[29]
6	9.60	$C_{17}H_{24}O_{11}$	404.1319	232	405.1402 $[M+H]^+$/−2.63	403.1253 $[M-H]^-$/−1.77	405/265, 233, 184	403/371, 223, 179	Secoxyloganin	[4]
7	11.79	$C_{21}H_{22}O_9$	418.1264	232, 276	419.1348 $[M+H]^+$/−2.73	417.1201 $[M-H]^-$/−2.38	419/257	417/255	Liquiritin[a]	[30]
8	11.92	$C_{26}H_{30}O_{13}$	550.1686	231, 276	551.1774 $[M+H]^+$/−2.69	549.1629 $[M-H]^-$/−2.79	551/257	549/255	Liquiritigenin-4'-apiosyl (1-2)-glucoside	[30]
9	12.08	$C_{27}H_{30}O_{16}$	610.1534	255, 350	611.1623 $[M+H]^+$/−2.69	609.1477 $[M-H]^-$/−2.61	611/465, 303	609/301	Rutin[a]	[1]
10	12.25	$C_{21}H_{20}O_{12}$	464.0955	232, 328	465.1041 $[M+H]^+$/−2.90	463.0895 $[M-H]^-$/−2.80	465/289	463/287, 175, 151	Not identify	–
11	12.33	$C_{21}H_{20}O_{12}$	464.0955	232, 328	465.1039 $[M+H]^+$/−2.47	463.0894 $[M-H]^-$/−2.59	465/447, 303, 289	463/287, 175, 151	Not identify	–
12	12.51	$C_{21}H_{20}O_{12}$	464.0955	258, 350	465.1040 $[M+H]^+$/−2.69	463.0891 $[M-H]^-$/−1.94	465/303	463/301	Quercetin-3-*O*-*β*-D-glucoside	[4]
13	12.62	$C_{21}H_{20}O_{11}$	448.1006	260, 345	449.1090 $[M+H]^+$/−2.59	447.0942 $[M-H]^-$/−2.04	449/287	447/285	Lluteolin-7-*O*-*β*-D-glucoside[a]	[4]
14	12.75	$C_{21}H_{18}O_{12}$	462.0798	254, 345	463.0882 $[M+H]^+$/−2.38	461.0735 $[M-H]^-$/−2.06	463/287	461/285	Not identify	
15	13.59	$C_{27}H_{30}O_{15}$	594.1585	270, 350	595.1675 $[M+H]^+$/−2.95	593.1526 $[M-H]^-$/−2.37	595/449, 287	593/285	Lonicerin	[4]
16	13.70	$C_{25}H_{24}O_{12}$	516.1268	326	517.1352 $[M+H]^+$/−2.22	515.1201 $[M-H]^-$/−1.16	517/499, 319, 163	515/353, 173, 191, 335	4,5-di-*O*-CQA[a]	[29]
17	14.10	$C_{25}H_{24}O_{12}$	516.1268	326	517.1354 $[M+H]^+$/−2.61	515.1207 $[M-H]^-$/−2.33	517/499, 319, 163	515/353, 191, 179	3,5-di-*O*-CQA[a]	[29]
18	15.15	$C_{25}H_{24}O_{12}$	516.1268	326	517.1351 $[M+H]^+$/−2.03	515.1204 $[M-H]^-$/−1.74	517/499, 319, 163	515/353, 203, 173, 255	3,4-di-*O*-CQA[a]	[29]
19	15.28	$C_{22}H_{22}O_{11}$	462.1162	258, 350	463.1248 $[M+H]^+$/−2.84	–	463/301	–	Not identify	–
20	15.45	$C_{40}H_{70}O_8$	678.5071	–	679.5151 $[M+H]^+$/−1.11	723.5076 $[M+HCOO]^-$/−3.43	679/661, 643	723/677	Not identify	–
21	15.90	$C_{26}H_{30}O_{13}$	550.1686	250	551.1775 $[M+H]^+$/−2.88	549.1625 $[M-H]^-$/−2.06	551/419, 257	549/417, 297, 255	Licuraside	[30]
22	16.28	$C_{21}H_{22}O_9$	418.1264	245, 360	419.1346 $[M+H]^+$/−2.25	417.1199 $[M-H]^-$/−1.90	419/257	417/255	Isoliquiritin	[30]
23	16.99	$C_{15}H_{12}O_4$	256.0736	238, 275	257.0811$[M+H]^+$/−1.03	255.0667 $[M-H]^-$/−1.63	257/239, 211, 163, 147	255/153, 135, 119	Liquiritigenin	[30]
24	17.31	$C_{23}H_{48}O_8$	452.3349	–	453.3435 $[M+H]^+$/−2.89	–	453/435, 390, 340, 210	–	Not identify	–
25	17.77	$C_{28}H_{32}O_{14}$	592.1792	334	593.1880 $[M+H]^+$/−2.56	591.1731 $[M-H]^-$/−1.98	593/447, 285	591/283	Linarin[a]	[31]
26	18.02	$C_{15}H_{10}O_6$	286.0477	255, 344	287.0554 $[M+H]^+$/−1.35	285.0410 $[M-H]^-$/−1.88	287/153	285/241, 199, 175, 151	Luteolin[a]	[4]
27	18.74	$C_{22}H_{22}O_{10}$	446.1213	268, 348	447.1295 $[M+H]^+$/−2.08	491.1206 $[M+HCOO]^-$/−2.47	447/285	491/283	Not identify	–
28	19.82	$C_{65}H_{106}O_{32}$	1398.6667	–	1399.6781 $[M+H]^+$/−2.93	1397.6632 $[M-H]^-$/−2.68	1399/1021, 897, 751	1397/1073, 911, 749	Macranthoidin B[a]	[4]
29	20.25	$C_{59}H_{96}O_{27}$	1236.6139	–	1237.6247 $[M+H]^+$/−2.85	1235.6099 $[M-H]^-$/−2.65	1237/1193, 759, 631	1235/911, 663, 641	Macranthoidin A[a]	[4]
30	20.73	$C_{53}H_{86}O_{22}$	1074.5611	–	1075.5713 $[M+H]^+$/−2.74	1073.5569 $[M-H]^-$/−2.89	1075/967, 863	1073/911, 749, 583	Dipsacoside B[a]	[4]
31	21.60	$C_{44}H_{64}O_{18}$	880.4093	248	881.4192 $[M+H]^+$/−3.02	879.4045 $[M-H]^-$/−2.85	881/603, 539, 399	879/861, 351	Not identify	–
32	23.20	$C_{42}H_{62}O_{17}$	838.3987	248	839.4085 $[M+H]^+$/−3.01	837.3935 $[M-H]^-$/−2.48	839/663, 487, 469, 451	837/661, 351	Licorice-saponin G_2	[19]
33	23.28	$C_{42}H_{60}O_{16}$	820.3881	248	821.3971 $[M+H]^+$/−2.06	819.3829 $[M-H]^-$/−2.49	821/779, 637, 391, 265	819/643, 351	Licorice-saponin E_2	[30]
34	24.59	$C_{42}H_{62}O_{16}$	822.9321	246	823.4133 $[M+H]^+$/−2.72	821.3986 $[M-H]^-$/−2.54	823/647, 471, 453, 435	821/351	Glycyrrhizic acid [a]	[30]
35	25.78	$C_{42}H_{62}O_{16}$	822.9321	246	823.4134 $[M+H]^+$/−2.84	821.3989 $[M-H]^-$/−2.91	823/647, 471, 453, 435	821/351	Isomeric glycyrrhizic acid	[30]
36	26.59	$C_{53}H_{86}O_{22}$	1074.5610	–	1075.5711 $[M+H]^+$/−2.56	1073.5566 $[M-H]^-$/−2.61	1075/728	1073/911, 749, 603	Macranthoside B	[4]
37	27.55	$C_{21}H_{20}O_6$	368.1260	384	369.1341 $[M+H]^+$/−2.27	367.1193 $[M-H]^-$/−1.60	369/341, 327, 313	367/309, 297	Glycycoumarin	[30]

[a] The identity was confirmed by comparing its t_R, UV spectra, ESI–TOF–MS and ESI–IT–MS data with those of the reference substances.

Table 3
Linear-regression data, LODs, LOQs, precision, accuracy and recovery of the 15 major constituents as determined by RRLC–MS/MS.

Analyte	Regression equation	r^2	Linear range (μg/L)	LOD (μg/L)	LOQ (μg/L)	Precision RSD[a] (%)		Accuracy[b] (%)		Standard addition recovery[c] (%) mean ± SD (n = 3)
						Intra-day	Inter-day	Intra-day	Inter-day	
5-*O*-CAQ	$y = 950.0x - 340.6$	0.9976	500–50,000	100.0	333.3	3.3	3.9	98.7	98.2	98.9 ± 3.3
3-*O*-CAQ	$y = 2071x - 732.4$	0.9994	500–50,000	38.0	126.6	2.1	3.3	101.2	102.6	103.3 ± 2.8
4-*O*-CAQ	$y = 1044x - 377.7$	0.9996	500–50,000	146.5	488.3	2.6	3.7	99.2	99.7	99.3 ± 3.0
Liquiritin	$y = 3777x - 584.0$	0.9998	100–50,000	15.4	51.3	3.8	4.7	97.6	97.1	96.7 ± 2.4
Rutin	$y = 1269x + 110.0$	0.9996	50–50,000	10.8	36.1	3.2	4.3	98.7	99.3	98.4 ± 2.9
Luteolin-7-*O*-β-D-glucoside	$y = 3436x + 767.7$	0.9940	100–50,000	16.9	56.2	3.5	4.2	97.0	96.6	97.6 ± 2.0
4,5-di-*O*-CQA	$y = 751.2x - 126.4$	0.9994	500–50,000	40.0	133.3	2.2	3.7	102.8	102.1	104.8 ± 4.1
3,5-di-*O*-CQA	$y = 1541x - 116.4$	0.9996	100–50,000	21.4	71.4	2.9	4.8	100.2	99.7	102.1 ± 2.5
3,4-di-*O*-CQA	$y = 2431x - 645.4$	0.9988	100–50,000	13.5	45.0	2.5	3.1	101.3	102.2	103.6 ± 3.4
Luteolin	$y = 5387x - 446.9$	0.9996	50–50,000	4.7	15.5	3.3	3.9	100.8	99.3	99.7 ± 4.0
Linarin	$y = 344.9x + 28.58$	0.9994	500–50,000	111.9	373.1	4.1	5.3	96.1	95.3	94.3 ± 3.3
Macranthoidin B	$y = 511.5x - 38.58$	0.9976	50–50,000	1.8	6.0	3.4	4.8	98.9	97.3	96.6 ± 3.2
Macranthoidin A	$y = 748.5x - 22.70$	0.9986	50–50,000	2.2	7.4	3.9	5.1	96.6	95.9	95.2 ± 2.1
Dipsacoside B	$y = 871.7x - 59.88$	0.9988	50–50,000	1.7	5.6	3.6	5.3	97.3	96.5	95.7 ± 3.0
Glycyrrhizic acid	$y = 1277x - 33.50$	0.9996	50–50,000	4.3	14.5	2.9	3.8	100.3	99.8	98.9 ± 3.5

[a] RSD, relative standard deviation.
[b] Accuracy (%) = 100 × mean of measured concentration/nominal concentration.
[c] The data are presented as the average of three determinations, where standard addition recovery (%) = 100 × (amount found − original amount)/amount spiked.

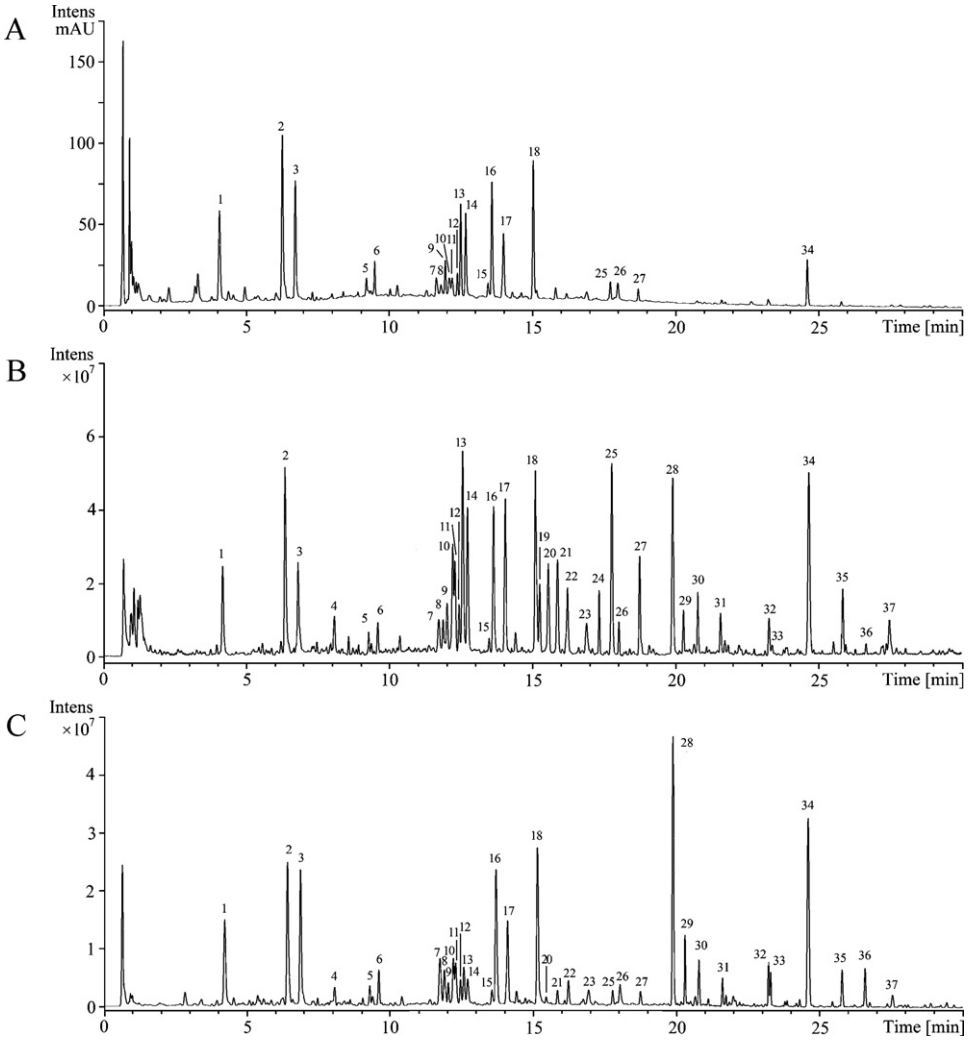

Fig. 2. Representative chromatograms of the DHT granule extract. (A) DAD at 254 nm. (B) BPC in positive-ion ESI-IT-MS. (C) BPC in negative-ion ESI-IT-MS.

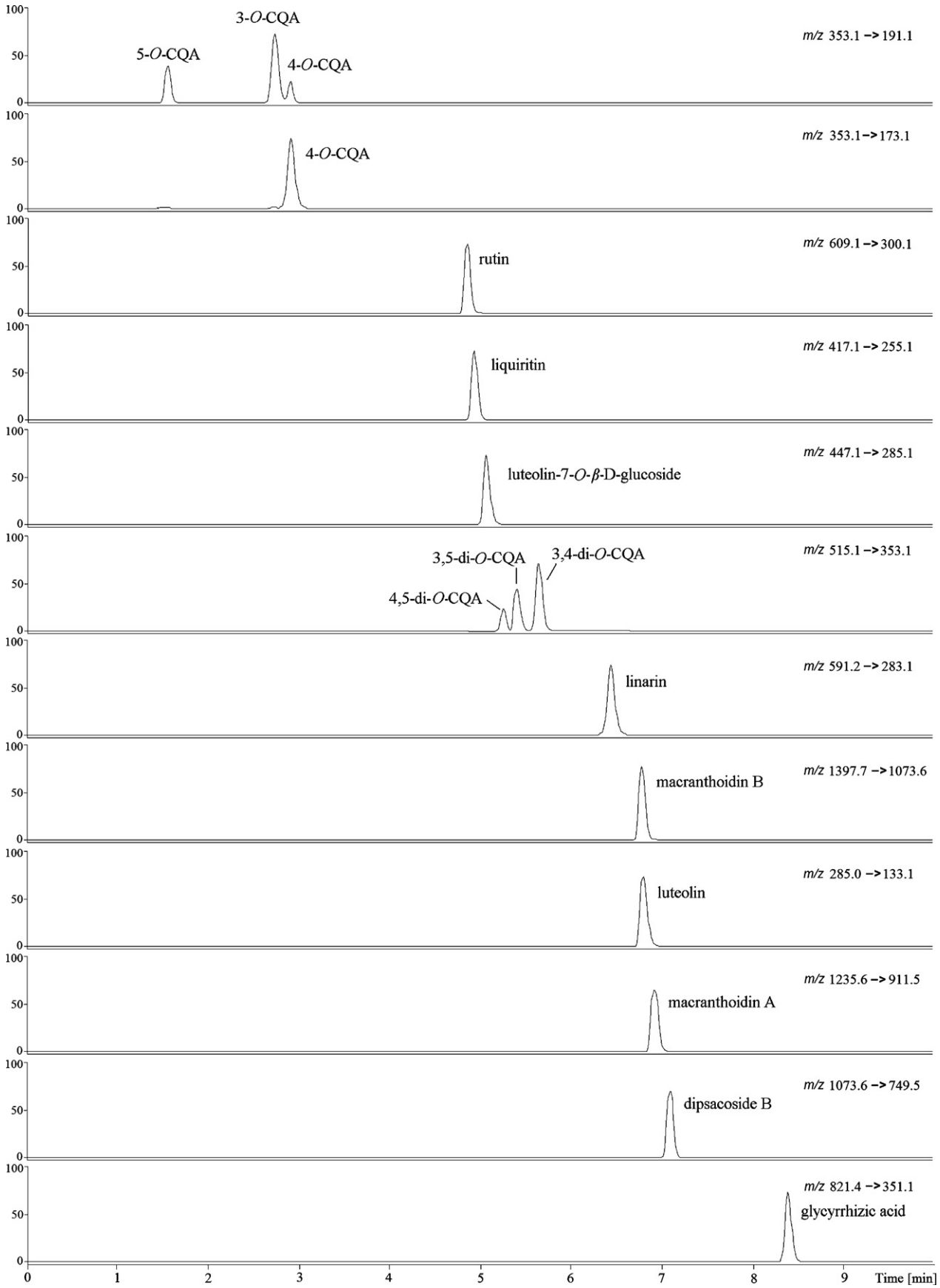

Fig. 3. RRLC chromatograms obtained by MRM for the negative-ion ESI triple quadrupole MS of the 15 reference substances.

Table 4
Contents of the 15 major constituents in 16 batch of DHT granule samples (mean ± SD, $n = 3$, μg/g).

Analyte	D10909	D11011	D11013	D11014	D11015	D11016	D11017	D11101	D21006	D21007	D21008	D21009	D21010	D21011	D21101	D21102
5-*O*-CAQ	471.8 ± 14.2	492.2 ± 13.9	570.6 ± 18.1	622.2 ± 21.7	626.4 ± 20.9	551.9 ± 17.8	556.3 ± 16.6	457.8 ± 13.5	511.4 ± 17.5	571.0 ± 17.2	608.6 ± 19.6	490.3 ± 12.1	641.3 ± 18.2	470.0 ± 13.4	536.7 ± 18.3	577.6 ± 17.1
3-*O*-CAQ	808.3 ± 25.3	776.2 ± 27.2	980.6 ± 39.3	1055.5 ± 42.5	1072.1 ± 37.6	1237.3 ± 43.1	1013.2 ± 32.5	721.0 ± 22.3	842.1 ± 30.3	1112.9 ± 37.7	1044.5 ± 34.6	763.3 ± 25.6	1293.4 ± 42.9	1013.4 ± 37.5	988.6 ± 33.8	966.0 ± 35.6
4-*O*-CAQ	414.4 ± 16.9	431.8 ± 16.2	513.9 ± 18.4	546.7 ± 19.9	546.7 ± 17.6	466.5 ± 15.4	506.7 ± 18.9	432.3 ± 15.1	469.4 ± 16.1	463.7 ± 14.9	485.4 ± 17.7	422.0 ± 14.3	552.9 ± 17.1	395.9 ± 13.7	486.3 ± 16.8	543.5 ± 18.3
Liquiritin	91.1 ± 4.1	139.2 ± 5.4	139.4 ± 4.9	151.9 ± 5.7	151.7 ± 5.3	152.5 ± 5.9	105.9 ± 4.3	70.7 ± 3.1	155.3 ± 6.5	150.3 ± 4.9	172.3 ± 6.2	121.6 ± 3.5	170.5 ± 7.1	157.3 ± 6.2	112.1 ± 4.7	71.3 ± 3.5
Rutin	56.3 ± 2.2	56.3 ± 2.1	51.6 ± 1.9	48.2 ± 1.9	55.8 ± 1.9	66.8 ± 2.4	65.4 ± 2.2	36.4 ± 1.3	48.4 ± 2.0	57.5 ± 2.4	61.7 ± 2.3	58.9 ± 2.1	68.3 ± 2.6	63.0 ± 2.4	58.9 ± 2.1	37.1 ± 1.2
Luteolin-7-*O*-β-D-glucoside	140.6 ± 6.3	132.5 ± 5.1	123.0 ± 4.1	129.2 ± 4.3	138.0 ± 5.3	123.5 ± 4.5	147.7 ± 6.6	70.4 ± 2.9	106.4 ± 4.8	121.6 ± 4.9	125.8 ± 4.2	135.2 ± 5.3	121.2 ± 4.8	112.4 ± 5.3	138.0 ± 5.7	87.7 ± 3.7
4,5-di-*O*-CQA	324.8 ± 10.8	338.5 ± 11.6	468.6 ± 14.9	459.4 ± 14.1	461.7 ± 14.3	406.7 ± 12.9	379.3 ± 12.7	347.7 ± 12.3	399.9 ± 12.6	392.3 ± 12.7	412.4 ± 12.1	314.1 ± 9.5	469.5 ± 17.3	332.5 ± 10.6	378.2 ± 11.5	457.1 ± 16.2
3,5-di-*O*-CQA	252.5 ± 9.5	279.2 ± 10.2	331.3 ± 11.1	385.6 ± 12.9	382.3 ± 13.5	413.2 ± 13.6	338.7 ± 11.2	204.1 ± 7.7	268.2 ± 10.1	363.6 ± 10.6	374.6 ± 11.4	289.4 ± 10.5	428.6 ± 16.1	336.0 ± 10.9	333.3 ± 11.3	268.6 ± 9.9
3,4-di-*O*-CQA	369.4 ± 11.3	380.1 ± 12.2	472.6 ± 15.1	522.3 ± 17.8	504.3 ± 16.9	518.9 ± 18.1	465.7 ± 17.4	366.4 ± 12.5	420.7 ± 13.6	490.5 ± 14.9	501.1 ± 16.1	376.2 ± 11.7	581.6 ± 17.3	432.4 ± 13.8	452.6 ± 14.7	471.3 ± 15.3
Luteolin	50.1 ± 2.3	52.6 ± 2.4	53.8 ± 2.1	55.1 ± 2.5	59.4 ± 2.7	54.0 ± 2.4	57.5 ± 2.6	26.7 ± 1.3	36.7 ± 1.8	45.3 ± 2.1	44.3 ± 1.9	47.7 ± 2.0	51.8 ± 2.3	36.3 ± 1.6	50.4 ± 2.2	33.4 ± 1.5
Linarin	49.4 ± 2.4	93.2 ± 4.3	146.2 ± 6.9	146.6 ± 6.7	131.9 ± 5.6	152.8 ± 7.0	44.4 ± 2.1	262.2 ± 11.9	159.4 ± 7.6	170.9 ± 8.1	182.2 ± 8.0	79.9 ± 3.7	198.5 ± 8.6	161.6 ± 6.8	70.1 ± 3.1	251.6 ± 11.3
Macranthoidin B	1232.2 ± 40.9	1336.4 ± 49.2	1719.5 ± 65.6	1728.9 ± 57.3	1660.7 ± 68.5	1732.5 ± 76.1	1448.7 ± 50.4	1315.3 ± 49.5	1263.6 ± 45.3	1682.2 ± 71.2	1557.1 ± 58.9	1033.7 ± 36.8	1888.9 ± 79.7	1302.9 ± 45.7	1317.4 ± 46.3	1570.6 ± 52.9
Macranthoidin A	109.1 ± 4.2	1115.3 ± 4.7	146.4 ± 6.1	149.8 ± 5.6	140.2 ± 5.2	146.7 ± 5.3	106.6 ± 3.8	142.9 ± 4.7	113.7 ± 4.5	154.0 ± 6.0	145.8 ± 4.2	99.0 ± 3.9	168.1 ± 6.7	118.1 ± 4.3	123.8 ± 4.6	179.2 ± 6.5
Dipsacoside B	132.0 ± 4.8	145.6 ± 5.2	179.5 ± 7.1	190.0 ± 7.8	199.6 ± 7.9	195.0 ± 6.6	139.1 ± 5.4	139.7 ± 5.1	149.3 ± 6.1	202.3 ± 8.7	188.1 ± 7.2	106.5 ± 4.7	209.5 ± 8.2	159.0 ± 6.8	152.0 ± 6.0	181.0 ± 7.6
Glycyrrhizic acid	330.6 ± 12.2	335.6 ± 11.3	299.4 ± 10.2	380.6 ± 13.1	390.3 ± 14.2	354.5 ± 12.6	328.5 ± 13.8	229.9 ± 7.9	361.2 ± 11.3	324.7 ± 10.9	367.9 ± 11.2	375.3 ± 12.3	392.5 ± 13.3	374.3 ± 12.5	313.2 ± 11.4	181.3 ± 6.3

4. Conclusions

In this study, a method based on RRLC–MS was developed for the identification and determination of the major constituents in DHT granules. The separation speed and efficiency of RRLC was high, and a full separation of the 37 major constituents and a quantitative determination of the 15 major constituents in the DHT granules were completed within 30 min and 10 min, respectively. RRLC coupled with TOF–MS and IT–MS quickly identified or tentatively characterized 28 compounds in the DHT granules based on their determined exact molecular weights and fragmentation patterns. With the quantitative analytical method, RRLC–MS/MS was simple, fast, and showed good linearity, precision and recovery for determination of the 15 major constituents in the DHT granules. Furthermore, the developed RRLC–MS/MS method was applied for the quality evaluation of the 16 batches of DHT granule samples, and the results indicated that the qualities of the 16 DHT samples were stable. Therefore, our proposed method could be suitable for use in the quality control of DHT granules.

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