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A targeted analysis of flavonoids in asparagus using the UPLC-MS technique

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Abstract: Production development for asparagus has become an important research subject due to its low shelf life. In order to determine the content of flavonoids in asparagus tips and shoots, LC-MS-based method was performed for a targeted analysis of flavonoids in asparagus, and 34 peaks attributed to the targeted flavonoids were characterised. Twelve peaks corresponding to rutin, isoquercitrin, quercetin, naringin, taxifolin, vitexin, genistin, daidzein, luteolin, chrysin, and kaempferide were identified and quantified from the asparagus tips and shoots by the LC-MS-based detection with monitoring of parent/daughter ions. The results showed that rutin (> 99%) was the main flavonoid present in the asparagus tips and shoots. Although the tips and shoots contained almost similar compounds, the content of the major compounds, especially rutin, was significantly different. Therefore, the method established through this study could be used for quantitative analysis of flavonoids, especially rutins, in asparagus. The result will provide a theoretical basis for food development in asparagus.

Keywords: asparagus; flavonoids; targeted analysis; LC-MS

Asparagus (*Asparagus officinalis* L.) is a valuable perennial crop known for its edible and medicinal properties. In China, asparagus has been used as an optional medicine for resisting cough, fungal infection, inflammation, arteriosclerosis, and cancer (Wang et al. 2013; Fan et al. 2015). Moreover, China is the largest producer and exporter of asparagus. Asparagus is an important source of bioactive compounds, and its consumption has been increasing (Zhang et al. 2018a). In addition, the extraction of active ingredients, especially flavonoids (Wang et al. 2011), from asparagus has been widely attempted. It is probable that the flavonoids in green asparagus are the most abundant phenolics and the main bioactive compounds (Tang et al. 2014). Reports have shown that the flavonoids possess antioxidant, anticancer, arteriosclerosis prevention, and immunity enhancing properties (Fuentes-Alventosa et al. 2009b; Negi et al. 2010; Lee et al. 2014).

LC-MS methods has been used for identification of flavonoids Lee et al. 2005. Compared with the established quantitative methods such as spectrophotometry, capillary electrophoresis, and immunofluorescence, this method has the advantages of better specificity, resolution and accuracy, and lower disturbance. Additionally, in the past years, many studies on the flavonoids in asparagus have mainly focused on their extraction process (Zhang et al. 2018a, 2018b), total content (Ku et al. 2018), and pharmacological and

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antioxidant effects (Lee et al. 2018). However, the quantitative analysis of flavonoids has been investigated to a lesser extent (Tang et al. 2014). Targeted analysis is widely used for the quantitative analysis of known metabolites in biological samples (Lu et al. 2008). Therefore, LC-MS-based method is valuable to analyse the active compounds in asparagus, especially flavonoids. This is the first study to conduct quantitative analysis of flavonoids in the tips and shoots of locally grown asparagus using LC-MS-based targeted technique. The results of our study will provide a basis for the further flavonoid food development in asparagus.

MATERIAL AND METHODS

Chemicals and reagents. Methanol (Wokai, chromatography grade), plastic centrifuge tube (5 mL, QSP), catechin (Aladdin, ≥ 97%; remifene, > 96%), dihydromyricetin (remifene, > 98%), puerarin (Aladdin, ≥ 98%), daidzein (remifentine, > 98%), taxifolin (remifentine, > 98%), vitexin (remifentin, > 98%), isovitexin (remifentin, > 97%), luteolin (remifentin, > 98%), rutin (Aladdin, > 98%), isobutyl quercetin (remifentin, > 98%), naringin (Aladdin, > 98%), genioside (remifentin, > 98%), myricetin (remifentin, > 98%), quercitrin (remifentine, > 98%), fisetin (Sigma-Aldrich, 98%), astragalin (remifentine, > 98%), diosmine (Aladdin, ≥ 95%), liquiritigenin (remifentz, > 98%), daidzein (Aladdin, > 98%; remifentz, > 98%), baicalin (Aladdin 98%), quercetin (remifense, > 98%), naringin (source leaves, 98%), luteolin (Aladdin, > 98%), genistein (Wokai, 98%), silybin (Sigma-Aldrich, ≥ 98%), kaempferol (Aladdin, > 98%), apigenin (remifense, > 98%), icariin (Aladdin, > 98%), formononetin (remifentin, > 98%), biochanin A (remifentin, > 98%), chrysin (Aladdin, > 98%), and kaempferide (remifentin, > 98%). [The detailed information is listed in the supplementary material available online (ESM) – specifically in Table S1].

Instruments. Triple quadrupole mass spectrometer (AB Sciex API 4000; AB SCIEX, USA), ultra-performance liquid chromatography system (ACQUITY UPLC; Waters, USA), a vortex mixer (Qilinbeirer QL-866; Zhejiang instrument equipment, China), ultrasonic cleaning machine (Shumei kw-100tdv; Dongguan City South Nekon Machinery, China), 0.22μm PTFE membrane filter (JinTeng, Suzhou renaud biotechnology, China), refrigerated centrifuge (H1650-w, Shanghai zhaodi biological science, China), and vacuum concentrator (Eppendorf 53050; Eppendorf, Germany).

Plant material. Asparagus samples were collected, which have been planted for four years, from the experimental fields of Heze juxinyuan food (China). They were harvested, wrapped in aluminium foil and frozen in liquid nitrogen. The fresh plant tissue was ground to a fine powder with liquid nitrogen and stored at –80 °C for further analysis. Six replicates of each sample were used.

Preparation of the standard solution. The standards were accurately weighed and dissolved in methanol to prepare the stock solution. Then, 34 individual standards were diluted to the required concentration to construct standard curve, respectively. All the standard solutions were stored at –80 °C for detection. [The detailed information is listed in ESM – specifically in Table S2].

Flavonoid extraction. Flavonoids were extracted as reference to Jaegle et al. (2016). Each sample, consisting of 100 mg of the freeze-dried material, was extracted with 5 mL of 100% methanol. The samples were blended in a Sorvall Omnimixer, model 17106 (DuPont, USA) at the maximum speed for 1 min and then filtered through a filter paper. The extracts were stored at –20 °C until further analysis by UPLC. The extracts of each sample were prepared in six replicates. Sample pretreatment: 100 mg of the sample in a 5 mL centrifuge tube was treated with 2 mL 70% methanol solution and ultrasonicated for 60 min at 25 °C. The solution was then centrifuged for 3 min at 4 000 rpm and the supernatant was filtered through a 0.22 μm membrane filter into a bottle, and then directly injected into the UPLC unit.

UPLC-MS detection. The quantitative analysis of flavonoids was carried out using the triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI). The flavonoids were separated by UPLC through the C18 chromatographic column (2.1 × 100 mm, 1.7 μm; Waters, USA). The sample (5 μL) was injected into the chromatographic column with the column temperature maintained at 40 °C. Mixture of formic acid in water (0.1%) and acetonitrile was used as mobile phases A and B, respectively. The flow rate was set to 0.25 mL min^{-1}. The gradient elution conditions were (a) 0–1min, 10% B (b) 1–3 min, 10–33% B (c) 3–5 min, 33% B (d) 5–7 min, 33–90% B (e) 7–8.5 min, 90% B (f) 8.5–10 min, 90–10% B, and (g) 10–13 min, 10% B. The MS conditions were set for the electrospray ionization (ESI) source and negative ionization mode. The ion source temperature was 500°C, ion source voltage of –4500 V, collision gas of 6 psi, curtain gas of 30 psi, and atomised and auxiliary gas of 50 psi. The scan was performed using the multiple reaction monitoring (MRM).

Statistical analysis. The statistical analysis was performed on excel. The data is presented as numbers

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for the categorical variables, and the final data is expressed as mean ± standard deviation (SD). The significance was determined by *t*-test for SPSS 10.0.

RESULTS AND DISCUSSION

To accurately identify the ion pairs (parent/daughter ion pair) of the 34 standards (Table S1), LC-MS was used for the quantitative analysis of individual standards of flavonoids. Four mass spectrometer parameters, declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP), were optimised for good monitoring of MRM transitions and detection of determined compouds. As shown in Table 1, there was a proportional relationship between DP and CE, while CXP was adjusted to different values in each sample. Therefore, the results of the parent/daughter ion pair analysis gave the qualitative analysis of the flavonoid compounds.

To achieve the desired separation, we carried out chromatographic analysis of the standard solutions with analytes injection under the above optimised conditions. As shown in Figure 1, all the analytes had retention times between 2.6 and 7.7 min, and the intensity between $0.5 \times e^4$ and $1.07 \times e^5$ cps. Altogether, standard solutions with sample showed good separation of the compounds, no material interference, stable baseline, and good specificity in 10 min.

As described in the experimental, we measured the standard curve concentration of each standard analytes. As shown in Table 2, different concentration gradients, ranging from 6 to 10 concentration gradients, was set for each compound. The highest concentration was set to 800 ng mL^{-1}, and the lowest concentration was set to 0.02 ng mL^{-1} based on their concentration in plants.

The plot of concentration of the standard solutions vs the peak area was prepared from the concentrations of the standard solutions determined by LC-MS. The linear regression equation obtained for each standard compound is presented in Table 2. The correlation coefficient (r) was greater than 0.99. The limit of quantification (LOQ) was determined by the SNR (Signal to Noise Ratio) method, where the signal measured from the known low concentration sample was compared with that from the blank sample when the SNR was 10 : 1.

The flavonoid content of 12 asparagus tip and shoot samples were measured from the peak area, determined by LC-MS. Among the 34 targeted flavonoids, 12 flavonoids were identified and quantified from the 12 samples (Figure 2, Table 3, and Table S3). They include rutin, isoquercitrin, quercetin, naringin, taxifolin, vitexin, genistin, daidzein, luteolin, chrysin, kaempferide and quercetin-3-O-glucoside. Studies have reported to have identified some of the flavonoids, such as quercetin, kaempferol, isorhamnetin, nicotiflorin, narcisin, quercetin-3-O-glucoside, and quercetin-diglucoside, from asparagus (Nindo et al. 2003; Wang et al. 2011;

Table 1. Optimal condition of MS instrument for MRM monitoring of 34 targeted analytes

No.	Test material	Parent ion /daughter ion	DP	EP	CE	CXP
1	Catechin	288.968/109.0	−75	−10	−36	−7
2	Puerarin	415.076/267.1	−85	−10	−42	−5
3	L-Epicatechin	288.822/108.8	−45	−10	−32	−11
4	Dihydromyricetin	318.842/192.7	−75	−10	−12	−25
5	Daidzin	460.858/252.7	−50	−10	−22	−5
6	Vitexin	431.300/282.9	−95	−10	−40	−1
7	Rutin	609.192/300.0	−100	−10	−52	−9
8	Isovitexin	431.005/310.9	−80	−10	−30	−5
9	Cynaroside	447.060/284.6	−85	−10	−34	−7
10	Quercetin-3-glucoside	463.151/300.0	−50	−10	−36	−7
11	Taxifolin	302.799/284.9	−45	−10	−16	−5
12	Naringin	579.198/271.1	−115	−10	−44	−7
13	Astragalin	447.120/283.9	−75	−10	−34	−15
14	Diosmin	607.188/299.0	−105	−10	−38	−3
15	Quercitrin	446.954/299.9	−60	−10	−30	−5
16	Genistin	431.000/268.0	−85	−10	−46	−1
17	Myricetin	317.196/150.8	−70	−10	−34	−3
18	Fisetin	284.744/134.9	−75	−10	−30	−1
19	Baicalin	445.098/269.0	−70	−10	−27	−8
20	Daidzein	252.977/132.0	−100	−10	−54	−9
21	Liquiritigenin	254.856/119.1	−45	−10	−42	−19
22	Glycitein	282.980/267.8	−75	−10	−24	−5
23	Luteolin	284.873/132.9	−90	−10	−46	−11
24	Quercetin	301.255/150.6	−85	−10	−28	−11
25	Icariin	513.241/365.9	−135	−10	−36	−9
26	Silybin	480.996/124.9	−90	−10	−40	−7
27	Apigenin	268.908/116.8	−60	−10	−46	−1
28	Naringenin	271.222/119.0	−40	−10	−24	−3
29	Genistein	268.775/132.8	−105	−10	−42	−9
30	Kaempferol	285.091/92.9	−105	−10	−46	−7
31	Formononetin	266.922/251.7	−50	−10	−24	−5
32	Chrysin	253.030/142.9	−90	−10	−40	−7
33	Kaempferide	299.171/284.0	−45	−10	−28	−13
34	Biochanin A	283.008/267.9	−115	−10	−28	−5

DP – declustering potential; EP – entrance potential; CE – collision energy; CXP – cell exit potential

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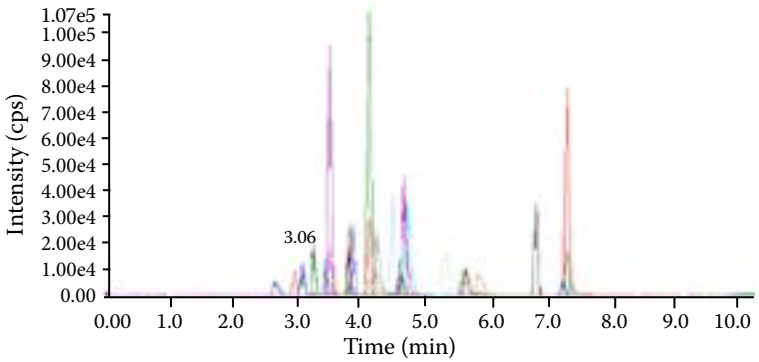

Figure 1. The chromatogram of the 34 targeted analytes obtained from the chromatographic analysis

Table 2. Regression equation and quantitative limit in each standard analyte

No.	Name	Regression equation	Correlation coefficient (r)	Linear range (ng mL^{-1})	Loq (ng mL^{-1})
1	Catechin	$y = 200x - 31.6$	0.9958	10.00–800.00	10
2	Puerarin	$y = 1\,310x - 135$	0.9959	1.25–100.00	1.25
3	L-Epicatechin	$y = 320x - 98.3$	0.9963	10.00–400.00	10
4	Dihydromyricetin	$y = 843x + 153$	0.9975	1.00–400.00	1
5	Daidzin	$y = 2\,480x + 327$	0.9952	0.50–100.00	0.5
6	Vitexin	$y = 736x + 29.7$	0.9975	1.00–400.00	1
7	Rutin	$y = 1\,070x + 490$	0.9911	0.50–200.00	0.5
8	Isovitexin	$y = 3\,690x - 377$	0.9964	0.40–400.00	0.4
9	Cynaroside	$y = 907x + 315$	0.9948	1.00–200.00	1
10	Quercetin-3-glucoside	$y = 1\,400x + 92.1$	0.9947	0.50–200.00	0.5
11	Taxifolin	$y = 2180x + 583$	0.9992	1.25–100.00	1.25
12	Naringin	$y = 527x + 378$	0.9984	2.00–400.00	2
13	Astragalin	$y = 855x + 101$	0.9948	1.00–400.00	1
14	Diosmin	$y = 1\,970x - 312$	0.9974	0.50–200.00	0.5
15	Quercitrin	$y = 506x + 122$	0.9945	1.00–200.00	1
16	Genistin	$y = 4\,400x - 212$	0.9955	0.25–100.00	0.25
17	Myricetin	$y = 745x - 1100$	0.9935	4.00–800.00	4
18	Fisetin	$y = 2\,500x - 863$	0.9988	0.80–800.00	0.8
19	Baicalin	$y = 1\,250x - 125$	0.9968	2.00–400.00	2
20	Daidzein	$y = 2\,030x - 188$	0.9954	0.40–25.00	0.4
21	Liquiritigenin	$y = 3\,370x + 631$	0.9986	1.00–200.00	1
22	Glycitein	$y = 6\,200x + 332$	0.9971	0.20–40.00	0.2
23	Luteolin	$y = 4\,640x + 901$	0.9970	0.50–200.00	0.5
24	Quercetin	$y = 1\,950x - 748$	0.9979	1.00–400.00	1
25	Icariin	$y = 231x - 265$	0.9982	6.25–100.00	6.25
26	Silybin	$y = 2\,160x + 531$	0.9988	1.00–200.00	1
27	Apigenin	$y = 5\,810x + 821$	0.9954	1.00–40.00	1
28	Naringenin	$y = 1\,010x + 483$	0.9949	1.00–200.00	1
29	Genistein	$y = 1\,340x + 477$	0.9987	1.00–200.00	1
30	Kaempferol	$y = 377x + 405$	0.9950	4.00–800.00	4
31	Formononetin	$y = 26\,500x + 306$	0.9968	0.02–20.00	0.02
32	Chrysin	$y = 1\,300x + 173$	0.9905	0.50–100.00	0.5
33	Kaempferide	$y = 14\,100x - 299$	0.9957	0.10–100.00	0.1
34	Biochanin A	$y = 14\,000x + 442$	0.9979	0.05–20.00	0.05

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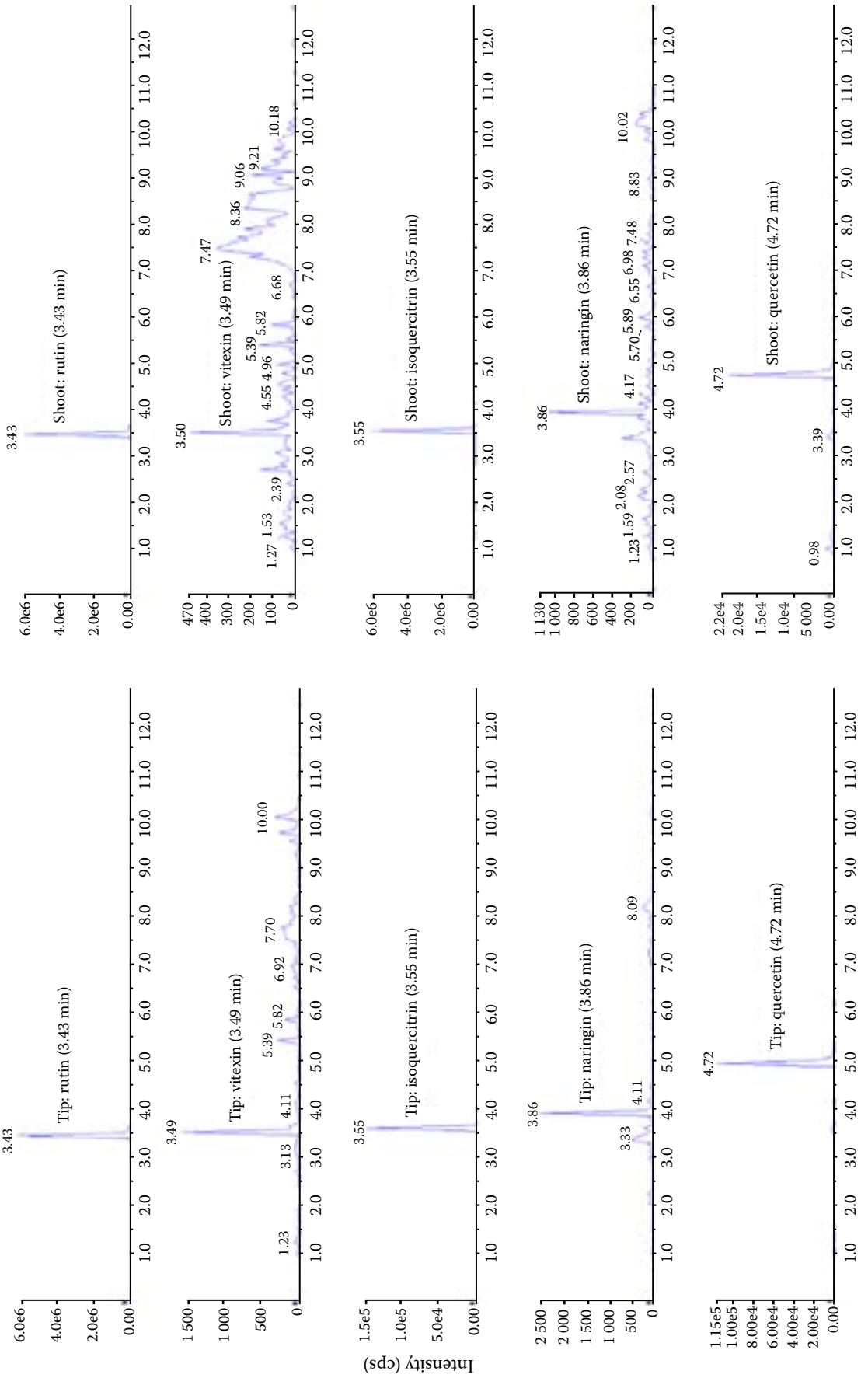

Figure 2. Five representative chromatograms of 12 identified flavonoids in asparagus tips (left) and shoots (right), including rutin,vitexin, isoquercitrin, naringin and quercetin

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Table 3. Detected flavonoid contents in asparagus tips and shoots

No.	Name	RT (min)	Shoot contents (μg g^{-1})	Tip contents (μg g^{-1})	Flavonoids type	Molecular formula
1	Rutin	3.43	173.19 ± 36.35	473.83*± 8.56	Flavonols	$C_{27}H_{30}O_{16}$
2	Isoquercitrin	3.55	0.47 ± 0.10	7.80*± 1.09	Isoflavones	$C_{21}H_{20}O_{12}$
3	Quercetin	4.12	0.19 ± 0.09	4.25*± 1.51	Flavonols	$C_{15}H_{10}O_7$
4	Naringin	3.8	ND	0.35 ± 0.04	Dihydroflavone	$C_{27}H_{32}O_{14}$
5	Taxifolin	3.8	ND	0.19 ± 0.05	Dihydroflavone	$C_{15}H_{12}O_7$
6	Vitexin	3.49	0.05 ± 0.005	0.16*± 0.05	Flavonols	$C_{21}H_{20}O_{10}$
7	Genistin	3.86	0.14*± 0.08	0.03 ± 0.01	Isoflavones	$C_{15}H_{10}O_5$
8	Daidzein	4.47	ND	0.06 ± 0.02	Isoflavones	$C_{15}H_{10}O_4$
9	Luteolin	4.66	0.06 ± 0.01	0.04 ± 0.03	Flavonoids	$C_{15}H_{10}O_6$
10	Chrysin	7.17	0.02 ± 0.01	ND	Flavonoids	$C_{15}H_{10}O_4$
11	Kaempferide	7.23	0.003 ± 0.0008	ND	Flavonols	$C_{16}H_{12}O_6$
12	Quercetin-3-glucoside	3.55	0.48 ± 0.10	8.26 ± 1.17	Flavonoid	$C_{21}H_{20}O_{12}$

*represents extremely significant difference in *t*-test for SPSS 10.0; RT – retention time; ND – not detected

Hamdi et al. 2017). In addition, the total flavonoid content was roughly 486 μg g^{-1} and 174 μg g^{-1} of the fresh weight of the asparagus tips and shoots, respectively. Of all the identified compounds, rutin had the highest concentration of 99.54% (173.19 μg g^{-1}) and 97.43% (473.83 μg g^{-1}) in asparagus tips and shoots, respectively. These results were in agreement with the previous reports that have identified the total flavonoid content in asparagus between 400 and 700 mg kg^{-1} of the fresh weight (Fuentes-Alventosa et al. 2009a). As shown in Table 3 (and Table S3), among 34 targeted standards, only 12 flavonoids were identified in our study, 7 were identified from the asparagus tips and shoots, 3 from only the tips, and 2 from only the shoots. Other than rutin (97.43%), isoquercitrin (1.6%), and Quercetin-3-glucoside (1.79%) in the tips, the amounts of other flavonoids were quite low. Based on molecular weight and formula, these flavonoids could be divided into four types (Table 3). *T*-test showed that the contents of some flavonoids in the asparagus tips were significantly different from those in the asparagus shoots ($P < 0.01$). The results indicated that contents of some flavonoids in the asparagus tips, especially rutins, can be more suitable for functional food and beverage products. Therefore, we have found a simple and accurate method for the quantitative analysis of flavonoids, and for the detection of rutin that can be used for specific products.

CONCLUSION

In this study, we carry out a targeted LC-MS detection method with 34 standard flavonoids. Twelve flavonoids were identified for quantitative analysis in asparagus tips and shoots. Especially, the rutin is the most flavonoid contents in asparagus. The method in our study is simple, accurate, and applicable for flavonoid contents, especially rutins. The results will provide a theoretical basis for functional food development.

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