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UPLC-QTOF-MS Analysis on Isoflavones in Douchi

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Abstract: Douchi is a kind of traditional Chinese fermented soybean food. Ultra-high performance liquid chromatography quadrupole-time of flight mass spectrometry (UPLC-QTOF-MS) was applied to separate and identify 12 kinds of isoflavones in Douchi within 16 min. The chromatographic separation was carried out on an ACQUITY UPLC HSS T3 column with a gradient elution program where water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid were used as mobile phases. Detection by using electrospray ionization of positive ion mode was applied in the mass spectrometry. Isoflavones were identified by determining the accurate mass and referring to references in this study.

Key words: Douchi, fermented soybean food, ultra-high performance liquid chromatography quadrupole-time of flight mass spectrometry (UPLC-QTOF-MS), isoflavone, identification

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Introduction

Douchi is a traditional fermented food with nutritional value and functionality in China. Isoflavones, which have the functionalities of anti-cancer, anti-oxidation, preventing cardiovascular disease, preventing osteoporosis and improving menopause syndrome (Alam *et al*., 2017; Arjmandi and Smith, 2002; Hyun *et al*., 2012; Chin *et al*., 2016), are functional components in Douchi (Wang *et al*., 2007; Wu *et al*., 2017). The structures of isoflavones can be divided into isoflavone aglycones and glycoside isoflavones (Table 1)(Penalvo *et al*., 2004). Many methods have been developed for detecting isoflavones in foods in recent years (Ahn-Jarvis *et al*., 2017; Fernandes *et al*., 2017). For instance, ultraviolet spectrophotometry (Klejdus *et al*., 2005), high performance liquid chromatography (HPLC) (Sun *et al*., 2011) and high performance liquid chromatography-mass spectrometry (HPLC-MS) (Bórquez *et al*., 2013) are applied to analyze isoflavones in soybean. Within these technologies, UPLC-QTOF-MS has the advantages of high resolution, high sensitivity, full scan, etc (Ferrer *et al*., 2005; Thurman *et al*., 2005). It has been applied widely for the characterization and identification of isoflavones in various foods (Abrankó *et al*., 2015; Gampe *et al*., 2016; Lee *et al*., 2015). So far, there have been no reports about the application of UPLC-QTOF-MS on the study of isoflavones in Douchi. The present study therefore used this technology to identify 12 kinds of isoflavones in Douchi.

Materials and Methods

Reagents and materials

Methanol and acetonitrile of LC-MS grade were purchased from Thermo Fisher (Thermo Fisher Corp.,

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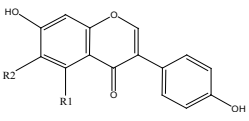
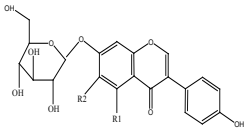
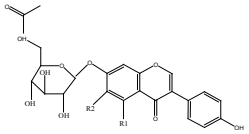
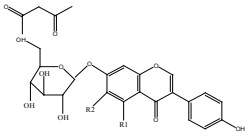
USA). Formic acid of LC-MS grade was obtained from Waters (Waters Corp., USA). Water was supplied by a Milli-Q purification system (Millipore Corp., France). Douchi samples were purchased from the supermarket. The 0.2 μm disposable membrane filters (Acrodisc® syringe filter with GHP membrane, 13 mm) were purchased from PALL (PALL Life Sciences, USA).

Table 1 Structures of isoflavones

Structure	R1	R2	Compound
	H	H	Daidzein
	OH	H	Genistein
	H	OCH_3	Glycitein
	H	H	Daidzin
	OH	H	Genistin
	H	OCH_3	Glycitin
	H	H	Acetyldaidzin
	OH	H	Acetylgenistin
	H	OCH_3	Acetylglycitin
	H	H	Malonyldaidzin
	OH	H	Malonylgenistin
	H	OCH_3	Malonylglycitin

Sample preparation

1.0 g of Douchi sample was accurately weighed and placed in a 50 mL test tube, 10 mL of methanol was added to this tube. The sample was extracted for 15 min under the ultrasonic condition, then centrifuged for 10 min at 10 000 rpm·min^{-1} (Hunan Herexi Instrument & Equipment Co., Ltd., China) and the supernatant was filtered through 0.2 μm membrane (PALL Life Sciences, USA) before injection.

UPLC-ESI-QTOF-MS conditions

ACQUITY UPLC I-Class tandem Xevo G2-S QTOF with UNIFI Screening platform (Waters Corp., USA) was used in this study. The LC separation was performed on the ACQUITY UPLC HSS T3 C18 column (2.1 mm×50 mm, ID 1.7 μm, Waters Corp., USA) and ACQUITY UPLC BHE C18 column (2.1 mm×100 mm, ID 1.7 μm, Waters Corp., USA) at 40℃ separately. Acetonitrile containing 0.1% formic acid and methanol containing 0.1% formic acid were tested as mobile phase B and water containing 0.1% formic acid (mobile phase A) was used for gradient elution of the analytical separation. The gradient elution program was as the followings: 0-1 min, 95% A; 1-12 min, 95% A -0% A; 12-13 min, 0% A; 13-13.01 min, 0% A -95% A and 13.01-16 min, 95% A. The flow rate of the mobile phase was 0.4 mL·min^{-1}.

The electrospray source was operated in the positive mode at 1.0 kV and the sample cone voltage was set at 20 V. The cone gas flow and the desolvation gas flow were 50 L·h^{-1} and 800 L·h^{-1}, respectively. Data were acquired in the range from 50 to 1200 mass-to-charge ratio (m/z). The source and desolvation temperature were set at 100℃ and 550℃, respectively.

Results

Fig. 1 showed the UPLC-QTOF-MS chromatogram of Douchi extract. Fig. 2 showed the MS^2 spectra of 12 kinds of isoflavones in the positive mode. These isoflavones were daidzin, glycitin, genistin, malonyldaidzin, malonylglycitin, acetyldaidzin, acetylglycitin, malonylgenistin, daidzein, acetylgenistin, glycitein and genistein.

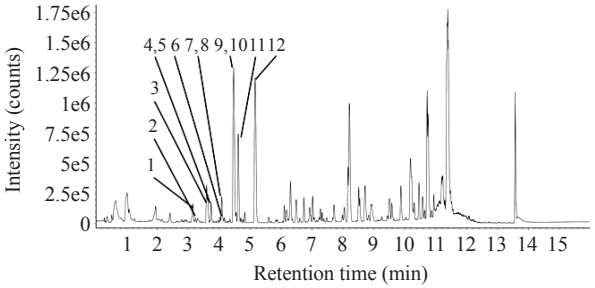


Fig. 1 UPLC-QTOF-MS chromatogram of Douchi extract

A total of twelve kinds of isoflavones in Douchi extract were analyzed in this study by UPLC-QTOF-MS (Figs. 1 and 2). Structures of the compounds were

provided by UNIFI software. The mass spectrometric characterization and retention time of 12 kinds of isoflavones in Douchi were summarized in Table 2.

In this study, the parent ions, a large number of fragment ions and their accurate molecular weight were determined. Meanwhile, a reliable elementary composition and structure identification data were provided by UNIFI software. Twelve different isoflavones in Douchi extract could be optimally separated in 16 min. The mass error between theoretical and observed molecular weight was less than 5 mDa.

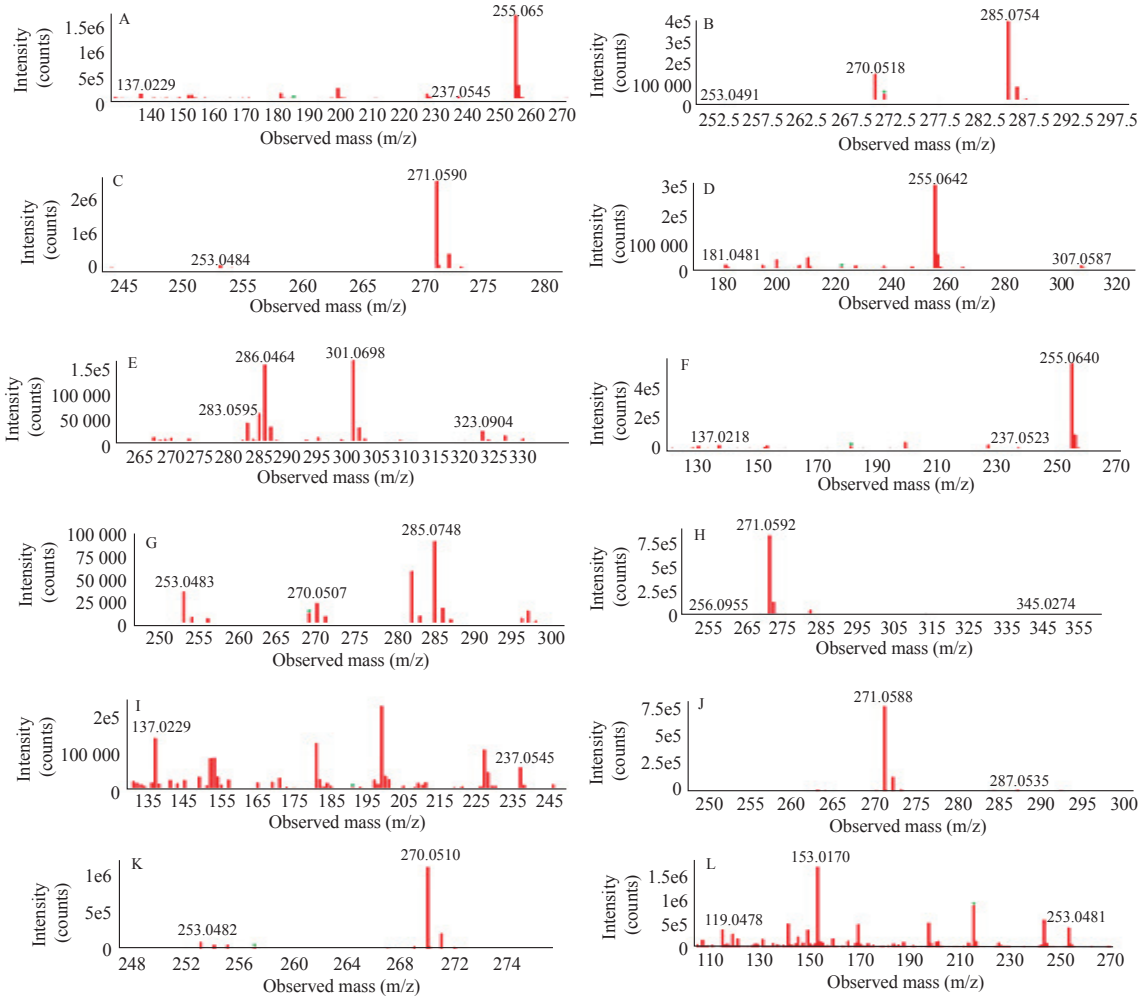


Fig. 2 MS^2 spectra of 12 kinds of isoflavones in positive mode

A, Daidzin; B, Glycitin; C, Genistin; D, Malonyldaidzin; E, Malonylglycitin; F, Acetyldaidzin; G, Acetylglycitin; H, Malonylgenistin; I, Daidzein; J, Acetylgenistin; K, Glycitein; L, Genistein.

Table 2 UPLC-QTOF-MS analysis of isoflavones in Douchi

Number	Component name	Formula	m/z	RT (min)	Observed neutral mass (Da)	Expected neutral mass (Da)	Fragment (Da)	Mass error (mDa)	Reference
1	Daidzin	$C_{21}H_{20}O_9$	417.1183	3.15	416.111	416.1107	137.0229, 237.0545, 255.065	0.2	John *et al.*, 2013
2	Glycitin	$C_{22}H_{22}O_{10}$	447.1285	3.23	446.1212	446.1213	270.0518, 285.0754, 253.0491	–0.1	Lee *et al.*, 2015
3	Genistin	$C_{21}H_{20}O_{10}$	433.1121	3.63	432.1048	432.1056	271.0590, 253.0484	–0.9	Abrankó *et al.*, 2015

Continued

Number	Component name	Formula	m/z	RT (min)	Observed neutral mass (Da)	Expected neutral mass (Da)	Fragment (Da)	Mass error (mDa)	Reference
4	Malonyldaidzin	$C_{24}H_{22}O_{12}$	503.1179	3.7	502.1107	502.1111	181.0481, 255.0642, 307.0587	–0.5	Lee *et al.*, 2015
5	Malonylglycitin	$C_{25}H_{24}O_{13}$	533.1286	3.73	532.1213	532.1217	286.0464, 283.0595, 301.0698, 323.0904	–0.4	Lee *et al.*, 2014
6	Acetyldaidzin	$C_{23}H_{22}O_{10}$	459.1282	4.09	458.1209	458.1213	255.0640, 237.0523, 137.0218	–0.4	Lee *et al.*, 2014
7	Acetylglycitin	$C_{24}H_{24}O_{11}$	489.139	4.09	488.1317	488.1319	285.0748, 270.0507, 253.0483	–0.2	Lee *et al.*, 2015
8	Malonylgenistin	$C_{24}H_{22}O_{13}$	519.1132	4.12	518.106	518.106	271.0592, 345.0274, 256.0955	–0.1	Lee *et al.*, 2015
9	Daidzein	$C_{15}H_{10}O_4$	255.0644	4.47	254.0571	254.0579	137.0229, 237.0545	–0.8	Lee *et al.*, 2015
10	Acetylgenistin	$C_{23}H_{22}O_{11}$	475.1227	4.54	474.1154	474.1162	271.0588, 287.0535	–0.8	Lee *et al.*, 2015
11	Glycitein	$C_{16}H_{12}O_5$	285.0749	4.62	284.0676	284.0685	270.0510, 253.0482	–0.9	Lee *et al.*, 2015
12	Genistein	$C_{15}H_{10}O_5$	271.0591	5.16	270.0519	270.0528	119.0478, 153.0170, 253.0481	–1	Lee *et al.*, 2015

Discussion

Effect of UPLC-ESI-QTOF-MS condition on analysis of isoflavones

The samples were measured in both positive and negative ESI modes to learn about the sensitivity of the method. Experiment results showed that the positive ion mode was more sensitive than the negative ion mode. Therefore, data acquired in the positive ionization mode were further used in this study.

ACQUITY UPLC BHE C18 and ACQUITY UPLC HSS T3 C18 columns were tested for separating compounds in this study. The results suggested that ACQUITY UPLC HSS T3 C18 column containing 1.7 μm HSS particles was more suitable for separating compounds in Douchi samples. Whether methanol or acetonitrile was selected as organic phase was studied and the results showed that acetonitrile presented better separation performance. The study on the addition of electrolyte showed that 0.1% formic acid in the mobile phase could improve peak shape and response as reported by Zhang *et al* (2013).

Analysis of isoflavones in Douchi

Peak 1 displayed a $[M+H]^+$ at m/z of 417.1183 and was characterized as daidzin. The loss of glucose molecule resulting in a fragment ion with m/z of 255.0650 (John *et al*., 2013). Then this fragment ion losing a molecule of H_2O formed the fragment ion with m/z of 237.0545. Then the fragment ion with m/z of 137.0229 was produced by retro Diels-Alder (rDA) reaction.

Peak 2 was identified as glycitin. Firstly, this compound lost a molecule of glucose to generate a fragment ion with m/z of 285.0754 (Lee *et al*., 2015). This ion lost a methyl group and an ion with m/z of 270.0518 was formed. Then this ion lost a hydroxyl group and formed an ion with m/z of 253.0491.

Peak 3 with m/z of 433.1121 corresponded to genistin. This ion produced ions at m/z of 271.0590 by losing a molecule of glucose and then an ion with m/z 253.0484 was formed by losing a molecule of H_2O (Abrankó *et al*., 2015).

Peak 4 was identified as malonyldaidzin with $[M+H]^+$ at m/z of 503.1179. Ion with m/z of 307.0587 was generated by the breakage of pyran and the loss

of hydroxyl in this ion. Fragment ion $[M+H]^+$ at m/z of 255.0642 corresponding to the loss of a malonyl group and a molecule of glucose (Lee *et al*., 2015). The broken of pyran, rDA reaction and the loss of oxygen contributed to the formation of ion with m/z of 181.0481.

Peak 5 with $[M+H]^+$ at m/z of 533.1286 was corresponding to malonylglycitin. The fragment with m/z of 283.0595 was generated by the loss of a malonyl group and a molecule of glucose (Lee *et al*., 2014). The breakage of pyran and the loss of one atom of oxygen in the malonylglycitin resulted in the occurrence of ion with m/z of 323.0904. Fragment with m/z of 301.0698 corresponded to the loss of two hydroxyl groups, a malonyl group and a phenol group. The ion with m/z of 286.0464 was formed by the loss of three hydroxyl groups, a malonyl group, a molecule of glucose and a phenol group.

Peak 6 corresponded to acetyldaidzin with m/z of 459.1282 (Lee *et al*., 2014). Its MS^2 fragment ion with m/z of 255.0640 was formed by the loss of a malonyl group and a molecule of glucose and then fragment ion with m/z of 237.0523 was generated by the loss of a molecule of H_2O, while fragment ion with m/z of 137.0218 was formed after rDA reaction.

Peak 7 showed a $[M+H]^+$ at m/z of 489.139 and it was identified as acetylglycitin, which lost a acetyl group and a molecule of glucose to form the fragment ion with m/z of 285.0748 (Lee *et al*., 2015). Fragment ion with m/z of 270.0507 was generated by the loss of a methyl group. And then the loss of one oxygen atom contributed to the formation of fragment ion with m/z of 253.0483.

Peak 8 was identified as malonylgenistin, which was corresponded with Lee *et al* (2015). This compound showed fragment ion with m/z of 345.0274 was formed by the breakage of pyran and the loss of a hydroxy group. The loss of a malonyl group and a molecule of glucose in malonylgenistin produced the fragment ion with m/z of 271.0592. The loss of a malonyl group, one atom of oxygen and a hydroxyl group as well as rDA reaction resulted in the formation of fragment ion with m/z of 256.0955.

Peak 9 showed a $[M+H]^+$ at m/z of 255.0644 and it was identified as daidzein. The fragment ion with m/z of 237.0545 was formed by the loss of a molecule of H_2O (Lee *et al*., 2015) and fragment ion with m/z of 137.0229 was generated by rDA reaction.

Peak 10 showed a positive ion with m/z of 475.1227, which was identified as acetylgenistin (Lee *et al*., 2015). Its MS/MS spectrum showed a fragment ion with m/z of 287.0535, which corresponded to the loss of an acetyl group, two hydroxyl groups and a phenol group. Fragment with m/z of 271.0588 was formed by the loss of an acetyl group and a molecule of glucose.

Peak 11 showed a $[M+H]^+$ at m/z of 285.0749, which generated fragment with m/z of 270.051 by the loss of a methly group (Lee *et al*., 2015). Fragment with m/z of 253.0482 corresponded to the loss of a methoxy group.

Peak 12 was identified as genistein (Lee *et al*., 2015). Its fragment ion with m/z of 253.0481 was generated by the loss of a molecule of H_2O and fragment ion with m/z of 153.0170 and 119.0478 were formed by rDA reaction.

Conclusions

A total of 12 kinds of isoflavones were separated and identified in Douchi extracts by UPLC-QTOF-MS within 16 min. A high resolution of UPLC-QTOF-MS analytical method was established in this study. The sensitivity together with mass accuracy of the UPLC-QTOF-MS would allow the unambiguous identification of isoflavones in fermented soybean foods. This method could be applied to the quality control of Douchi and the comparative analysis on quality differences among various fermented soybean foods.

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