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Determination of flavone *C*-glucosides in antioxidant of bamboo leaves (AOB) fortified foods by reversed-phase high-performance liquid chromatography with ultraviolet diode array detection

Yu Zhang[a], Bili Bao[a], Boyi Lu[a], Yiping Ren[b], Xiaowei Tie[b], Ying Zhang[a,*]

[a] *Department of Food Science and Nutrition, College of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou 310029, Zhejiang Province, PR China*
[b] *Zhejiang Provincial Center for Disease Prevention and Control, Hangzhou 310007, Zhejiang Province, PR China*

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Abstract

Reversed-phase high-performance liquid chromatography (RP-HPLC) with ultraviolet diode array detection (UV-DAD) was used for the simultaneous determination of four flavone *C*-glucosides, i.e. orientin, homoorientin, vitexin and isovitexin in several food systems fortified by the antioxidant of bamboo leaves (AOB), such as high temperature sterilized milk, sunflower seed oil and extruded rice cake for the first time. The method included extraction of flavone *C*-glucosides from AOB-fortified foods by methanol aqueous solution, deproteinating with saturated lead acetate solution and potassium oxalate, defatting with *n*-hexane and clean-up by solid-phase extraction (SPE) with Phenomenex C_{18} cartridges. Analytes were separated with Luna C_{18} 5 μm 250 mm × 4.6 mm column using acetonitrile and 1% (v/v) acetic acid (pH 3.0) as mobile phase. Good results were obtained with respect to repeatability (relative standard deviation (RSD) < 2.2%) and recovery (81.4–91.8%) which fulfilled the requirements defined by European Union (EU) legislation. The total amounts of four flavone *C*-glucosides were 12.56 μg/100 mL, 881.08 μg/100 mL and 1420.83 μg/100 g dry weight in AOB-fortified sterilized milk, sunflower seed oil and extruded rice cake, respectively. The method was successfully applied to the analysis of flavone *C*-glucosides in AOB-fortified samples. The optimized procedure could also be referenced for the separation of flavone *C*-glucosides in other fortified foodstuffs.

Keywords: Antioxidant of bamboo leaves (AOB); Flavone *C*-glucosides; RP-HPLC; Simultaneous determination

1. Introduction

Flavonoids, a large group of plant polyphenol secondary metabolites, are widely distributed in medicinal plants, fruit juices, teas and health beverage resulting in high human consumption [1]. More than 4000 different naturally occurring flavonoids have been identified [2] and the list is still growing. Although flavonoids have generally been considered to be non-nutritive agents, in recent years the health effects of flavonoids present in human diet have attracted much attention. Previous studies suggested that they acted as antioxidants [3,4], and there was also some evidence from epidemiological studies indicating an inverse association between the intake of flavonoids and the risk of cardiovascular diseases [5–7] and different types of cancer [8].

Flavone *C*-glucosides, an important constituent of the flavonoid family, were found in some plants, such as the tree *Pterocarpus marsupium* [9] and the fruits of *Cucurbitaceae* [10], etc. Various biological activities of flavone *C*-glucosides have been demonstrated including the antimicrobial activity of homoorientin (luteolin-6-glucoside) together with vitexin (apigenin-8-glucoside) against *Staphylococcus aureus*, *Bacillus subtilis* and *Pseudomonas aeruginosa* [11] and the protective effects of luteolin-7-glucoside against liver injury caused by carbon tetrachloride in rats, etc. [12].

* Corresponding author. Tel.: +86 571 8697 1388; fax: +86 571 8604 9803.
E-mail address: y_zhang@zju.edu.cn (Y. Zhang).

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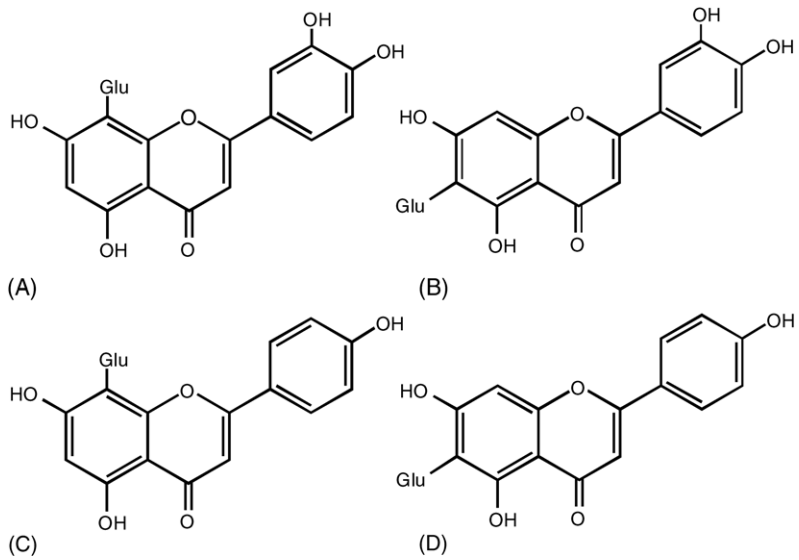

Fig. 1. Chemical structure of the studied flavone *C*-glucosides in antioxidant of bamboo leaves (AOB): (A) orientin; (B) homoorientin; (C) vitexin; (D) isovitexin. Glu: glucose.

Antioxidant of bamboo leaves (AOB), a pale brown powder extracted form bamboo leaves, was capable of blocking chain reactions of lipid autooxidation, chelating metal ions of transient state, scavenging nitrite compounds and blocking the synthetic reaction of nitrosamine testified by our previous study [13]. AOB has been listed in the national standards, i.e. GB2760, as a kind of food antioxidant in China. Additionally, it has the permission of being added into the puffed food, dairy, meat products and oils authorized by Ministry of Health, PR China [14]. The main functional components in AOB are flavonoids, lactones and phenolic acids. As for the flavonoids in AOB, flavone *C*-glucosides are a group of representative flavonoids in AOB reported by Zhang [15]. The chemical structures of four flavone *C*-glucosides, including orientin, homoorientin, vitexin and isovitexin found in AOB, are shown in Fig. 1.

Although there have been different approaches to their analysis, the separation and quantification of the flavone *C*-glucosides of a plant extract remains difficult, especially the simultaneous determination of different flavone *C*-glucosides in a sole analysis. HPLC is the method of choice for the analysis of polyphenolic-plant because of its versatility, precision and relatively low cost [16–18]. Most frequently, the method is used on reversed phase (RP) C_{18} columns, a binary solvent system containing acidified water, a polar organic solvent (acetonitrile or methanol), and diode array detection (DAD), which constitute a crucial and reliable tool in the routine analysis of plant phenolic compounds [19,20]. According to the most relevant bibliography, the HPLC-DAD chromatographic method seems to be a suitable tool for the separation and quantification of phenolic compounds in plant extracts [9]. To our knowledge, no other work on the identification and quantitative analysis of flavone *C*-glucosides from bamboo leaves by RP-HPLC-DAD has been previously reported. Therefore, optimizing and establishing the RP-HPLC-DAD method for the determination of flavone *C*-glucosides in AOB-fortified foods attracted our attention.

The aim of this study was to optimize and validate a reliable high-performance liquid chromatography method for the analysis of the flavone *C*-glucosides occurring in AOB-fortified food system such as the puffed food, dairy, meat products and oils.

2. Experimental

2.1. Samples

Antioxidant of bamboo leaves (AOB) was provided by Zhejiang University Innoessen Co., Ltd. (Hangzhou, China). AOB was prepared from the bamboo leaves of *Phyllostachys nigra* var. *henonis* identified by Research Institute of Subtropical Forestry of the Chinese Academy of Forestry (Hangzhou. China) [21]. Briefly, fresh bamboo leaves were collected during the autumn season in Anji district (Zhejiang Province, China) and air dried. The coarse powder of bamboo leaves was obtained after comminution and filtration (20–40 mesh) and 10 g powder was extracted with the time of 1 h by 100 mL 30% (v/v) ethanol aqueous solution using the hot reflux method. The filtrate was then isolated by membrane filtration to remove macro- and micro-molecular components such as polysaccharides and minerals and AOB was finally obtained after concentrating in vacuum and spray drying.

AOB-fortified high temperature sterilized milk was obtained from Anji Science Bio-product Co., Ltd. (Huzhou, China). AOB was added to milk before high temperature instantaneous sterilization with a fortification level of about 75 mg per liter milk. AOB-fortified sunflower seed oil was a gift from Shanghai Jiahe Food Co., Ltd. (Shanghai, China).

Ten gram AOB was dissolved in 40 g Span40 (heating if necessary) and 50 g Span80 was added into 9.9 kg sunflower seed oil in order to prepare 0.1% (m/m) oil-soluble AOB solution. The fortification level of AOB in sunflower seed oil was about 0.05%. AOB-fortified extruded rice cake was provided by Zhejiang Xiaowangzi Food Co., Ltd. (Hangzhou, China). Half a gram AOB was dissolved in 10 mL 95% (v/v) ethanol. A thousand gram palm oil mixed with this AOB solution was sprayed on the surface of extruded rice cake during the baking process at the temperature of 60 °C. The fortification level of AOB in extruded rice cake was about 0.03%.

2.2. Chemical reagents and materials

Acetonitrile and *n*-hexane (HPLC-grade) were provided by Tedia Company Inc. (OH, USA), and methanol was purchased from Merck & Co., Inc. (NJ, USA). Acetic acid from Lanxi Chemical Reagents Co. (Jinhua, China), ethanol, aluminum nitrate and sodium nitrite from Hangzhou Chang Zheng Chemical Factory (Hangzhou, China), lead acetate and potassium oxalate from Beijing Chemical Reagents Co. (Beijing, China) and *N*,*N*-dimethyl formamide from Hangzhou Shuang Lin Chemical Factory (Hangzhou, China) were all analytical grade reagents. Orientin (3′,4′,5,7-tetrahydroxyflavone-8-glucoside), homoorientin (3′,4′,5,7-tetrahydroxy-flavone-6-glucoside), vitexin (4′,5,7-trihydroxyflavone-8-glucoside) and isovitexin (4′,5,7-trihydroxyflavone-6-glucoside) standards (HPLC-grade) were obtained from Extrasynthese Company Inc. (Lyon, France). Lutin (3′,4′,5,7-tetrahydroxyflavone-3-*O*-lutinoside) standard (HPLC-grade) was provided by Sigma-Aldrich Co. (St. Louis, USA).

Water was purified with a Milli-Q system (Millipore, Bedford, USA). All solutions prepared for HPLC were passed through a 0.45 μm nylon filter before use.

2.3. Instrumentation

A vortex mixer WH-861 Minishaker (Taicang Science & Educational Instrumental Co., Jiangsu, China) was used to mix and homogenize AOB-fortified samples during pretreatment. An ultracentrifuge with cooling system (Beckman J-20-XP Series, Krefeld, Germany) was employed for sample defatting and protein removal. The pH of the buffer solutions and samples was adjusted with a PHS-3C precision pH meter (Leici Instrumental Co., Shanghai, China). Solid-phase extraction was carried out on a VacElut vacuum manifold for 20 cartridges (Varian, Palo Alto, CA, USA). The chromatographic system consisted of a HP-1100 series high-performance liquid chromatograph from Agilent Technologies (Palo Alto, CA, USA) equipped with a quaternary pump, on-line degasser, autosampler, automatic injector, column heater, and diode array detectors connected on-line.

2.4. Chromatographic conditions

Chromatographic separations of flavone *C*-glucosides were performed on a Luna C_{18} column (5 μm, 250 mm × 4.6 mm i.d.) protected by a RP18 guard column (5 μm, 4.0 mm × 3.0 mm i.d.), both from Phenomenex. A gradient programme was used with the mobile phase, combining solvent A (acetonitrile) and solvent B (1%, v/v, acetic acid adjusted to pH 3.0 with NaOH) as follows: 15% A (15 min), 15–40% A (10 min), 40% A (9 min), 40–15% A (6 min). The flow rate was 1.0 mL/min, the injection volume was 30 μL and the column temperature was maintained at 40 °C. Signal was monitored at 330 nm with the diode array detection.

2.5. Standard solutions and calibration curves

Orientin, homoorientin, vitexin and isovitexin stock solutions were prepared in methanol at the concentration of 850, 656, 640 and 544 μg/mL, taking into account the purity of the standards. These solutions were stored at 4 °C in the dark for less than 2 months and good stability of these analytes was demonstrated by comparison of their peak areas with those of fresh standard solutions. For quantitative analysis, matrix-matched calibration standards were prepared in triplicate at eight concentrations, 0.25–50 μg/mL for these four flavone *C*-glucosides.

2.6. Sample preparation

2.6.1. AOB-fortified high temperature sterilized milk

Aliquots (20 mL) of AOB-fortified high temperature sterilized milk were prepared and transferred into 50 mL polypropylene centrifuge tubes. Samples were shaken on a vortex mixer for 30 s and then allowed to stand at 4 °C in the dark, for at least 30 min, to enable sufficient equilibration with the milk matrix. After addition of 3 mL of saturated lead acetate solution and 3 mL of potassium oxalate, the samples were shaken again for 1 min and centrifuged at 10,000 × *g* for 10 min at 4 °C. The clear supernatant was carefully removed with the aid of a Pasteur pipette and further purified by solid-phase extraction, using Phenomenex C_{18} cartridges, previously activated by washing successively with methanol (twice with 3 mL). Prior to elution of the above supernatant, the cartridges were rinsed with 5 mL of methanol and 5 mL of Milli-Q water, respectively. Flavone *C*-glucosides were eluted from cartridges using 8 mL of *N*,*N*-dimethyl formamide (40% in methanol). The eluates were dried by nitrogen and set to a final volume of 4 mL with 50% methanol. Aliquots of 30 μL were directly injected into HPLC.

2.6.2. AOB-fortified sunflower seed oil

Aliquots (10 mL) of AOB-fortified sunflower seed oil were prepared and transferred into test tubes. The corresponding flavone *C*-glucosides were extracted with 10 mL of methanol by adding 2 mL salt solution for complete recovery [22,23].

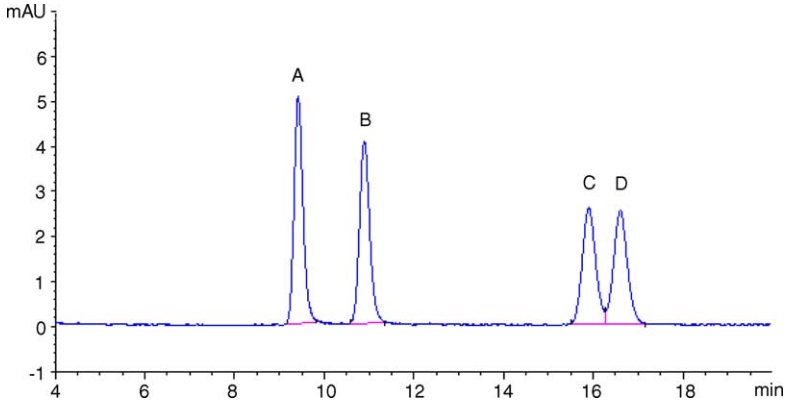

Fig. 2. Typical chromatograms of a four flavone *C*-glucoside standard mixture with ultraviolet diode array detection (UV-DAD): (A) homoorientin; (B) orientin; (C) vitexin; (D) isovitexin. Column: Luna C_{18}. Mobile phase: 1% (v/v) acetic acid (pH 2.7)—acetonitrile (gradient elution). Flow rate: 1 mL/min. Diode array detection at 330 nm. The other chromatographic parameters are described in Section 2.4.

Samples were defatted with 15 mL of *n*-hexane and shaken on a vortex mixer. The supernatant liquid was removed after 3 min and another aliquot (15 mL) of *n*-hexane was added for defatting again. The clear lower solution was carefully removed with the aid of a Pasteur pipette and diluted with the same volume of Milli-Q water. Then, samples were centrifuged at 10,000 × *g* for 10 min at 4 °C. For determination of percent of each component in the real sample, aliquots of 30 μL prepared sample were directly injected into the HPLC column.

2.6.3. AOB-fortified extruded rice cake

Aliquots (10 g) of AOB-fortified extruded rice cake triturated by mortar were weighted into 250 mL beaker. After adding 100 mL of 50% (v/v) methanol, samples were shaken by ultrasonic vibrator for 30 min and then deproteinated by

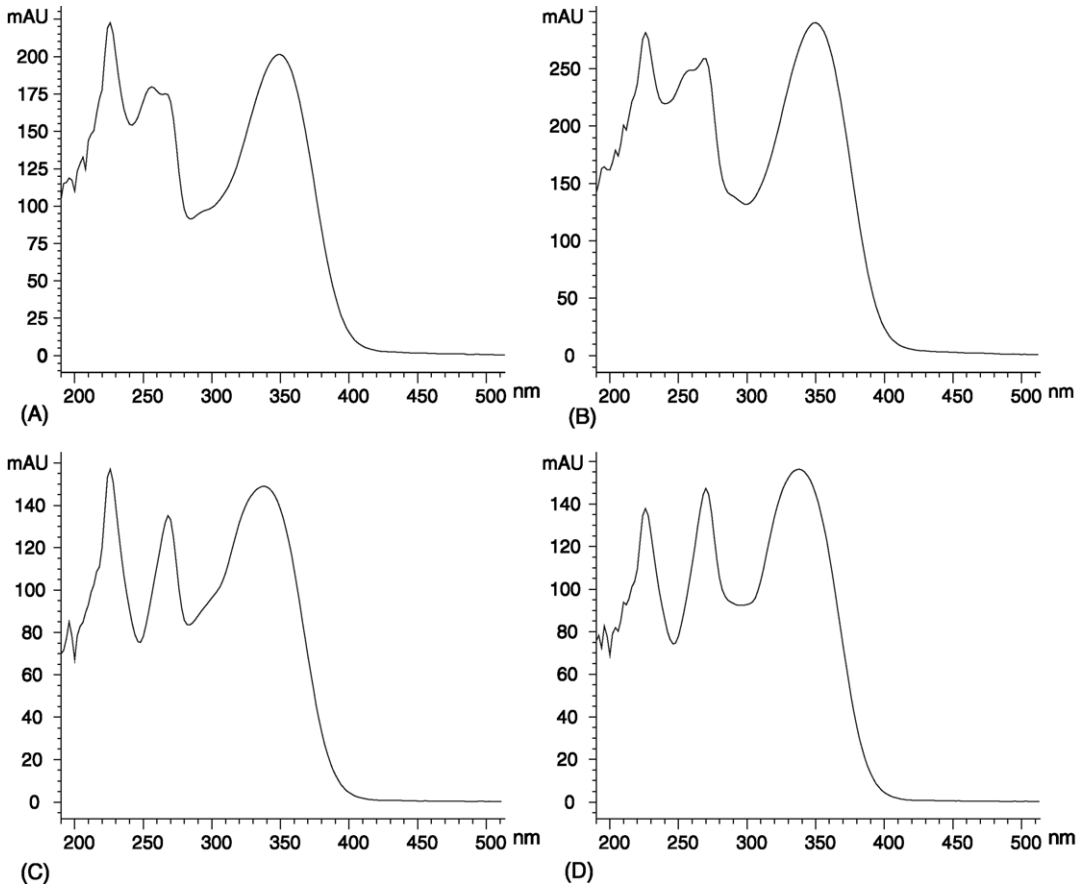

Fig. 3. The UV spectra of four detected flavone *C*-glucosides: (A) orientin; (B) homoorientin; (C) vitexin; (D) isovitexin.

Table 1
The effect of different levels of methanol as diluted solution on the response areas of the four flavone *C*-glucosides

Methanol level (%)	Response areas (mAU s)				
	Orientin	Homoorientin	Vitexin	Isovitexin	Total
0	119.6	44.2	25.0	70.1	258.9
20	124.6	44.5	24.8	74.2	268.1
40	149.7	52.8	30.0	92.7	325.2
50	141.1	59.9	28.2	87.2	316.4
60	132.5	55.4	24.8	82.7	295.4
80	101.8	44.9	20.0	64.4	231.1
100	63.6	23.6	20.4	35.7	143.3

precipitation using 5 mL of saturated lead acetate solution and 5 mL of potassium oxalate. Samples were allowed to stand at 4 °C for several minutes and 100 mL of salt solution was added for complete recovery. Five milliliter of clear supernatant was carefully removed with the aid of a Pasteur pipette and centrifuged at 10,000 × *g* for 10 min at 4 °C. Aliquots of 30 μL supernatant were directly injected into HPLC.

2.7. Quantification and identification

Quantification was performed on the basis of linear calibration plots of peak area against concentration. Calibration lines were constructed using a range of concentrations of the standard, selected such that concentration in the sample was at the middle of the range. Each line is based on eight concentrations of standard. Identification of the different compounds was made by comparison of their retention times with those of pure standards.

3. Results and discussion

3.1. Optimization of the HPLC conditions

A Luna C_{18} polar-endcapped column and gradient elution was used for the separation of flavone *C*-glucosides. Typical chromatograms corresponding to a standard mixture of the selected flavone *C*-glucosides (orientin, homoorientin, vitexin and isovitexin), using diode array detection, are shown in Fig. 2. The optimized HPLC conditions are described in Section 2.4. The *R*s values [29] for the four flavone *C*-glucosides on the Luna column were in all cases higher than 1.6. Unlike conventional C_{18} columns, Luna endcapped with hydrophilic (polar) functional groups enable separation of polar compounds in highly aqueous mobile phases, allowing a fast column equilibration for the gradient analysis. The UV spectra of four detected flavone *C*-glucosides were shown in Fig. 3. A good sensitivity was observed using diode array detection for all the flavone *C*-glucosides that was better monitored by its absorbance at 330 nm.

3.2. Optimization of sample pre-treatment

Several experiments were carried out in order to optimize the sample preparation and the chromatographic conditions. Optimal conditions for response areas of the flavone *C*-glucosides were investigated. It was proved that there were significant differences among response areas of flavone *C*-glucosides in different HPLC chromatograms when the flavone *C*-glucoside standards or AOB-fortified samples were dissolved and diluted with different levels of methanol. Preliminary assays suggested that the optimal level of methanol as dilution medium was 40–50% (v/v) according to total re-

Table 2
Calibration data of flavone *C*-glucoside standards

Compound	Linearity range (μg/mL)	Calibration equation[a]	LOD[b] (μg/mL)	LOQ[b] (μg/mL)	Correlation factor (R^2)
Orientin	0.2–50	$81.160C - 2.022$	0.03	0.09	0.9998
Homoorientin	1–50	$119.864C - 1.055$	0.02	0.06	0.9999
Vitexin	0.5–40	$84.965C + 4.000$	0.02	0.06	0.9995
Isovitexin	0.2–40	$99.410C + 4.350$	0.03	0.09	0.9996

[a] Eight data points ($n = 3$); *C*: the concentration of flavone *C*-glucosides (μg/mL).
[b] LOD = limit of detection; LOQ = limit of quantification.

Table 3
The repeatability data (μg/mL) from flavone *C*-glucoside standards and spiked samples[a]

Flavone *C*-glucosides[b]	Standards	Spiked samples[c]		
		S_1	S_2	S_3
Orientin	0.977 ± 0.009	0.814 ± 0.010	0.978 ± 0.005	0.860 ± 0.009
Homoorientin	0.983 ± 0.008	0.840 ± 0.015	0.979 ± 0.004	0.869 ± 0.019
Vitexin	0.978 ± 0.004	0.922 ± 0.010	0.910 ± 0.016	0.973 ± 0.003
Isovitexin	0.984 ± 0.005	0.891 ± 0.003	0.894 ± 0.010	0.920 ± 0.015

[a] Each value represents the mean ± SD for six different samples ($n = 6$). Flavone *C*-glucoside standard solutions were diluted to 1 μg/mL before the repeatability test. All of three blank samples were spiked at 1 μg/mL of mixed flavone *C*-glucoside standard solution.
[b] The retention time (means ± SD) of four flavone *C*-glucosides was determined during the whole repeatability test as follows: orientin, 11.21 ± 0.05 min; homoorientin, 9.73 ± 0.03 min; vitexin, 16.40 ± 0.06 min; isovitexin, 17.2 ± 0.03 min.
[c] S_1 = high temperature sterilized milk; S_2 = sunflower seed oil; S_3 = extruded rice cake.

sponse areas of the four flavone *C*-glucosides (Table 1). Considering the convenience of experimental operation, 50% of methanol was used to dissolve and dilute the standards and samples.

It is necessary to choose the suitable precipitator for protein removing during the pre-treatment of samples because the protein components in milk and extruded rice cake samples may interfere the quantitative determination of analytes and inevitably make trouble for the HPLC column. Therefore, the difficulty of extraction procedure increases especially when emulsifier is added into the food product, e.g. milk. Proteins in foodstuffs are usually removed using isoelectric precipitation by a mineral acid such as perchloric acid, HCl or H_2SO_4 at its isoelectric point during the pretreatment of samples. However, results in the present study indicated that isoelectric precipitation was not suitable for the precipitation of protein in these samples. Instead, saturated solution of lead acetate and 5% potassium oxalate were used.

Emulsification usually occurs under specific conditions especially in the present of fat matrix. In the present study, 50% (v/v) methanol aqueous solution was used for the extraction of flavone *C*-glucosides from AOB-fortified samples in order to elevate the response areas of analytes during the HPLC analysis. However, emulsification occurred unfortunately with the formation of colloid-stabilized emulsions because of the coexistence of fat matrix from samples, methanol and water. The formation of colloid-stabilized emulsions is an intractable problem that complicates the influencing factor of sample recovery. There are various methods for the demulsification of colloid-stabilized emulsions, such as electrosedimentation, supersonic demulsification [24], centrifugation and chemical demulsification [25,26]. The demulsification procedure in our study was done by adding NaCl into the water phase so that the emulsification process during sample pre-treatment was obviously inhibited and the high recovery of analytes was demonstrated according to the subsequent analysis.

3.3. Calibration and method validation

Four flavone *C*-glucoside standard solutions, in the concentration range 0.25–50 μg/mL, were prepared and analyzed. The calibration curves of the individual flavonoids were created by applying a statistical software of Chemstation for 3D. The peak area values (expressed in mAU·s) were plotted as average values of duplicate injections. The results of calibration were summarized in Table 2 and showed good linearity ($r > 0.999$) for all the compounds in the range of concentration tested (0.25–50 μg/mL) at 330 nm. Moreover, both the limit of detection (LOD) and limit of quantification (LOQ) were evaluated by the statistical software of Chemstation for 3D (Agilent Technologies, Palo Alto, USA).

The HPLC method was validated by defining the linearity, limits of quantification and detection (Table 2), peak purity, injection repeatability and recovery. For qualitative purposes,

Table 4
Intra-day recovery (%) and precision (RSD (%)) data from spiked AOB-fortified samples[a]

Analytes	AOB-fortified samples											
	High temperature sterilized milk				Sunflower seed oil				Extruded rice cake			
	S_0[b] (μg/mL)	S[b] (μg/mL)	Recovery (%)	RSD (%)	S_0[b] (μg/mL)	S[b] (μg/mL)	Recovery (%)	RSD (%)	S_0[b] (μg/mL)	S[b] (μg/mL)	Recovery (%)	RSD (%)
Oritentin	0.113 ± 0.008	0.928 ± 0.014	81.4	2.2	0.836 ± 0.008	1.814 ± 0.017	97.8	2.2	0.147 ± 0.003	1.007 ± 0.022	86.0	2.4
Homoorientin	0.297 ± 0.006	1.137 ± 0.020	84.0	2.6	1.778 ± 0.012	2.757 ± 0.024	97.9	2.1	0.311 ± 0.021	1.180 ± 0.004	86.9	2.1
Vitexin	0.124 ± 0.007	1.046 ± 0.009	92.2	1.3	0.218 ± 0.005	1.112 ± 0.021	89.4	2.3	0.038 ± 0.003	1.011 ± 0.006	97.3	1.0
Isovitexin	0.108 ± 0.005	0.999 ± 0.028	89.1	3.2	0.846 ± 0.004	1.756 ± 0.041	91.0	4.2	0.149 ± 0.003	1.069 ± 0.044	92.0	4.6

[a] The concentration data of analytes were shown as means ± SD ($n = 6$). All of three AOB-fortified samples were spiked at 1 μg/mL of mixed flavone *C*-glucoside standard solution.
[b] The concentration data of analytes in samples before spiking (S_0) and after spiking (S).

Table 5
Calibration data of flavone *C*-glucoside spiked samples[a]

Sample	Analytes	Linearity range (μg/mL)	Calibration equation[a]	Correlation factor (R^2)
High temperature sterilized milk	Orientin	0.08–2.5	80.535*C* − 0.944	0.999
	Homoorientin	0.08–2	116.021*C* − 1.882	0.998
	Vitexin	0.04–1.6	87.918*C* − 0.367	0.995
	Isovitexin	0.04–1.6	103.358*C* − 0.348	0.996
Sunflower seed oil	Orientin	0.08–2.5	82.134*C* + 0.934	0.997
	Homoorientin	0.08–2	114.342*C* − 1.870	0.999
	Vitexin	0.04–1.6	86.881*C* − 1.247	0.999
	Isovitexin	0.04–1.6	102.447*C* + 0.645	0.995
Extruded rice cake	Orientin	0.08–2.5	81.656*C* + 1.442	0.998
	Homoorientin	0.08–2	115.742*C* − 0.897	0.998
	Vitexin	0.04–1.6	86.971*C* + 1.145	0.994
	Isovitexin	0.04–1.6	102.658*C* + 0.457	0.996

[a] Eight data points ($n = 3$); *C*: the concentration of flavone *C*-glucosides (μg/mL).

the method was further evaluated by taking into account the precision of retention time and peak purity of analytes. A high repeatability in the retention time was obtained with RSD values lower than 1% for both standards and AOB-fortified food systems (Table 3). The peak purity was studied in the main four peaks representing four flavone *C*-glucosides in AOB. In no case were impurities or co-elutions were observed (match factor ≥98%).

The repeatability of the method was estimated for determination of both flavone *C*-glucoside standards and analytes of AOB-fortified foods. A standard solution (1 μg/mL) containing the four reference compounds was injected six times, and the RSD values were calculated for both the retention time and integration area. Good repeatability was obtained for the four flavone *C*-glucoside standards, orientin (0.977 μg/mL), homoorientin (0.983 μg/mL), vitexin (0.978 μg/mL) and isovitexin (0.984 μg/mL) with SD lower than 20 ng/mL. Corresponding blank samples, i.e. without AOB-fortified high temperature sterilized milk, sunflower seed oil and extruded rice cake, spiked at the concentration of 1 μg/mL were also extracted six times to evaluate the repeatability of the extraction process. The mean amount and SD values of each constituent were calculated (Table 3).

AOB-fortified sample solutions spiked at the concentration of 1 μg/mL were analyzed in order to evaluate the intra-day repeatability. The results were summarized in Table 4. As no previous study was tested for these four flavone *C*-glucosides in all of three food systems, 1 μg/mL was selected as a reference value for these compounds. Recoveries of 81–98% were obtained for these four flavone *C*-glucosides in all of three food systems with RSD lower than 4.6%. These results fulfilled the requirements defined by EU legislation [27]. The flavone *C*-glucosides were not so efficiently extracted from the sterilized milk. The multiplicity of milk constitutes and the complexity of extraction method affected the whole extraction process of AOB-fortified milk. Better recovery data were obtained for the determination of flavone *C*-glucosides in spiked sunflower seed oil compared to the other two foods studied (Table 4). Therefore, this HPLC method can be regarded as selective, accurate and precise.

3.4. Analysis of AOB-fortified samples

Matrix-matched calibration curves were prepared using raw milk, sunflower seed oil and extruded rice cake fortified with four flavone *C*-glucosides at different concentration levels. The results of calibration were summarized in Table 5 and showed good linearity ($r > 0.995$) for all the compounds in the range of concentration tested at 330 nm.

Fig. 4 shows the chromatograms of the extracts of three samples fortified with four flavone *C*-glucosides. The AOB-fortified milk chromatogram showed several non-identified peaks at retention times different from those of the analytes. These unknown compounds, which could not be easily removed according to Section 2.6.1, were present in the AOB-fortified milk extract. Fortunately, the interference of these peaks for the analytes could negligible because the retention time of these impurity peaks did not overlay with peaks of four flavone *C*-glucosides analyzed. Analysis of the AOB-fortified milk sample rendered concentrations

Table 6
Flavone *C*-glucosides in AOB-fortified samples[a]

AOB-fortified samples	Flavone *C*-glucosides				
	Orientin	Homoorientin	Vitexin	Isovitexin	Total
Sterilized milk (μg/100 mL)	2.22 ± 0.10	5.87 ± 0.15	2.37 ± 0.11	2.10 ± 0.10	12.56 ± 0.22
Sunflower seed oil (μg/100 mL)	201.08 ± 2.09	425.64 ± 2.68	51.84 ± 1.23	202.52 ± 1.34	881.08 ± 5.05
Extruded rice cake (μg/100 g dry weight)	327.43 ± 8.16	676.87 ± 29.86	84.7 ± 4.12	331.83 ± 11.42	1420.83 ± 34.66

[a] Each value represents the mean ± SD for six different samples ($n = 6$).

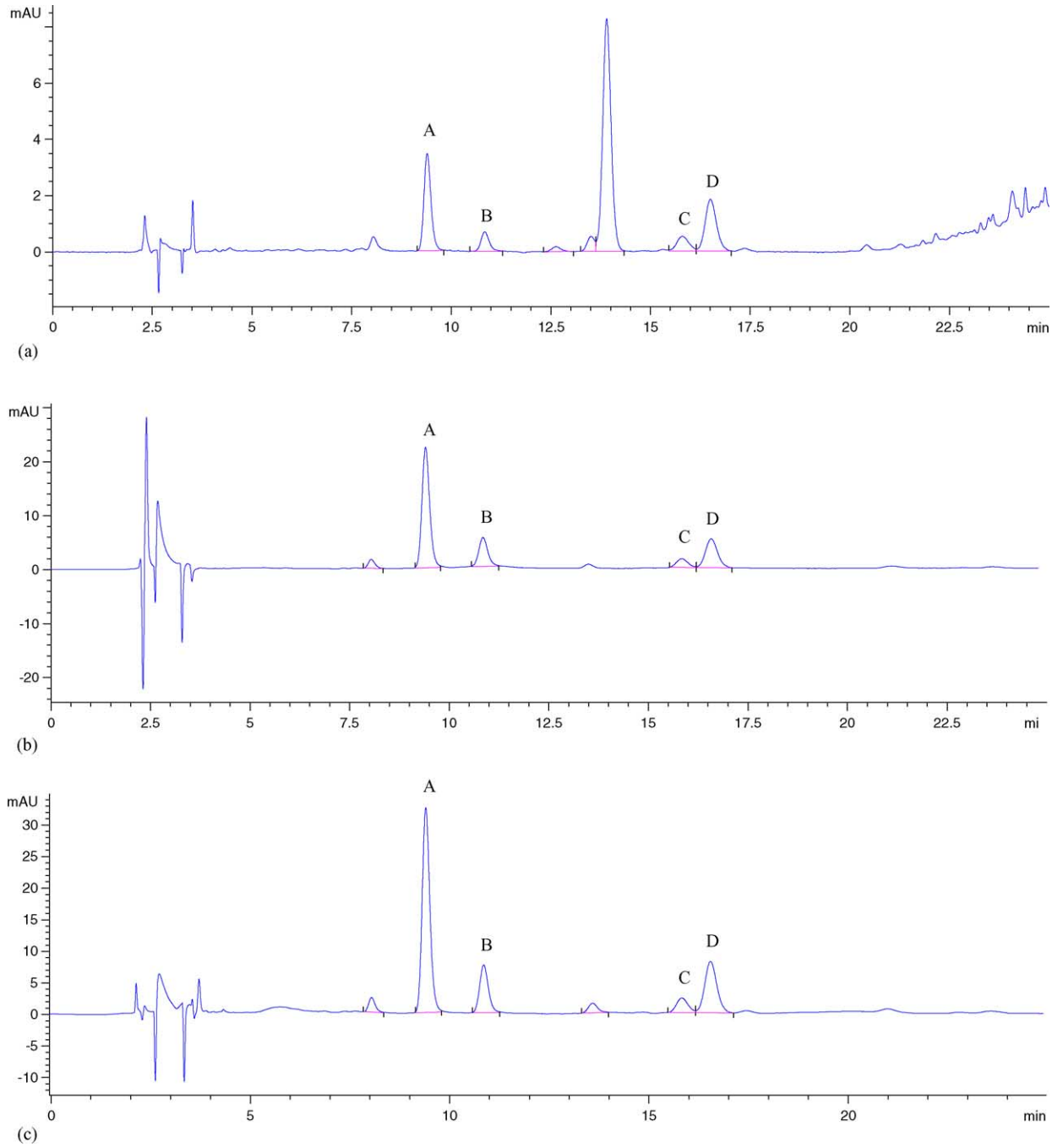

Fig. 4. Chromatograms with diode array detection of: (a) an extract of high temperature sterilized milk fortified with AOB; (b) an extract of sunflower seed oil fortified with AOB; (c) an extract of extruded rice cake fortified with AOB. (A) Homoorientin; (B) orientin; (C) vitexin; (D) isovitexin. Column: Luna C_{18}. Mobile phase: 1% (v/v) acetic acid (pH 2.7)—acetonitrile (gradient elution). Flow rate: 1 mL/min. Diode array detection at 330 nm.

of 2.22 μg/100 mL (RSD = 4.73%, $n = 6$), 5.87 μg/100 mL (RSD = 2.59%, $n = 6$), 2.37 μg/100 mL (RSD = 4.61%, $n = 6$) and 2.10 μg/100 mL (RSD = 4.74%, $n = 6$) for orientin, homoorientin, vitexin and isovitexin, respectively (Table 6). On the other hand, The AOB-fortified sunflower seed oil and extruded rice cake chromatograms showed clear peaks at corresponding retention times to those of the analytes. Few impurity peaks were detected during the whole HPLC analysis. These results indicated that the pre-treatment and extraction method for the above two AOB-fortified foods was more feasible and effective compared to that used for AOB-fortified milk. Furthermore, the detailed concentrations of analytes in these two AOB-fortified foods were summarized in Table 6. According to the EU legislation, the accuracy of a confirmatory method should be 80–110% for samples [28,29]. Recoveries in this study were 81–98%, with RSD of 1.0–4.6% which fulfilled the legislation requirements.

3.5. Conclusion

This work describes a HPLC methodology for the quantification of four flavone *C*-glucosides in AOB-fortified foods. The use of a polar endcapped Luna C_{18} column enables separation of four analytes without the need of ion-pair reagents. No column blocking problems were observed as described. The method is sensitive enough for the analysis of four flavone *C*-glucosides, with limits of quantification fulfilled the EU Legislation. Excellent levels of accuracy and precision were obtained for orientin, homoorientin, vitexin and isovitexin. Good results were obtained with respect to repeatability (relative standard deviation (RSD) < 2.2%) and recovery (81.4–91.8%). The total amounts of four flavone *C*-glucosides were 12.56 μg/100 mL, 881.08 μg/100 mL and 1420.83 μg/100 g dry weight in AOB-fortified sterilized milk, sunflower seed oil and extruded rice cake, respectively (Table 6). The method was successfully applied to the analysis of flavone *C*-glucosides in AOB-fortified sample. The optimized procedure could also be referenced for the separation of flavone *C*-glucosides in other fortified foodstuffs. Further work is in progress to extend the developed methodology to other food matrices and water samples.

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