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Determination of ascorbic acid and its related compounds in foods and beverages by hydrophilic interaction liquid chromatography

Akihiro Tai *, Eiichi Gohda

Department of Immunochemistry, Division of Pharmaceutical Sciences, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama 700-8530, Japan

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Abstract

A new hydrophilic interaction liquid chromatography method for the simultaneous determination of ascorbic acid (AA), erythorbic acid (EA), 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) and 2-*O*-β-D-glucopyranosyl-L-ascorbic acid (AA-2βG) was developed using a diol column with an isocratic solution of acetonitrile-66.7 mM ammonium acetate solution (85:15, v/v) at a detection wavelength of 260 nm. The calibration curves were found to be linear in the range of 1–50 μg/ml for AA and EA and in the range of 2.5–100 μg/ml for AA-2G and AA-2βG. Detection limits of AA, EA, AA-2G and AA-2βG were 0.3, 0.3, 0.03 and 0.03 μg/ml, respectively. This method was satisfactorily applied to the determination of AA, EA, AA-2G and AA-2βG in a fruit, a food and beverages. The results show that the procedure is simple and sensitive and that it can be employed for the simultaneous determination of AA and its related compounds in foods and beverages.
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1. Introduction

L-Ascorbic acid (AA), also known as vitamin C, has various physiological and pharmacological functions, including functions in collagen synthesis [1], intestinal absorption of iron [2] and drug metabolism [3], and acts as an important biological antioxidant [4]. Since humans, monkeys and guinea pigs cannot synthesize AA due to a lack of L-gulono-γ-lactone oxidase, they must take this essential nutrient from foods. AA is, apart from its role as a nutrient, an antioxidant commonly used for maintaining organoleptic quality in many food systems. D-Erythorbic acid (EA) (synonyms: D-isoascorbic acid, D-araboascorbic acid) is a C-5 epimer of AA. EA has chemical properties very similar to those of AA and is widely used as a food antioxidant in processed foods [5,6]. However, the antiscorbutic activity of EA is limited and has been reported to be only one-twentieth of that of AA in guinea pigs [7]. Except for high-performance liquid chromatography (HPLC) [8,9] and capillary electrophoresis [10] methodologies, the commonly used analytical procedures to measure vitamin C do not distinguish between the two isomers.

In 2004, 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G), a stable AA glucoside already used as a quasi-drug principal ingredient in skin care, was approved by the Japanese Government as a food additive. AA-2G is synthesized from AA and α-glucans by regioselective transglycosylation with cyclodextrin glucanotransferase from *Bacillus stearothermophillus* [11]. AA-2G is very stable in neutral solution and resistant to heat, light and oxidation. This vitamin C derivative is hydrolyzed to AA by α-glucosidase and exhibits physiological activities in vitro and in vivo [12–14]. AA-2G is considered to be promising as a pro-vitamin C agent in the field of foods and nutrition. Recently, AA-2G has been isolated as a naturally occurring compound from Kimchi, a Korean traditional fermented vegetable food [15,16]. A stereoisomer of AA-2G, 2-*O*-β-D-glucopyranosyl-L-ascorbic acid (AA-2βG), has also been isolated from *Lycium* fruit, a popular traditional Chinese food [17]. It has been reported that AA-2βG exhibits vitamin C activity and has a therapeutic effect in scorbutic ODS rats [18]. Thus, in addition to AA or EA as an antioxidant, these AA glucosides may simultaneously exist in foods. However, to the best of our knowledge, no HPLC method for simulta-

* Corresponding author. Tel.: +81 86 251 7962; fax: +81 86 251 7926.
E-mail address: atai@pharm.okayama-u.ac.jp (A. Tai).

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neous separation of AA, EA, AA-2G and AA-2βG has been reported.

Reversed-phase liquid chromatography (RPLC) is currently the most popular method in the field of HPLC. Although a powerful separation mode, a major limitation of RPLC is the lack of adequate retention of polar molecules. There is an alternative mode of chromatography in which a polar stationary phase, such as propylamine bonded to silica, is used with aqueous-organic mobile phases. This mode is similar to normal-phase chromatography in that polar compounds are retained longer than non-polar compounds and the polar mobile phase component, water, is a strong solvent. This method has been used for many years for polar compounds, particularly carbohydrates [19–21]. In 1990, Alpert used the term "hydrophilic interaction chromatography" (HILIC) to describe the use of a polar stationary phase with aqueous-organic mobile phases [22]. Therefore, the HILIC mode seems to be suitable for the analysis of AA and its related compounds with high polarity.

The aim of the present study was to develop a new HPLC method based on hydrophilic interaction chromatography for the separation of AA, EA, AA-2G and AA-2βG. The method was further applied for the simultaneous determination of AA and its related compounds in foods and beverages.

2. Experimental

2.1. Chemicals

2-*O*-α-D-Glucopyranosyl-L-ascorbic acid (AA-2G) and 2-*O*-β-D-glucopyranosyl-L-ascorbic acid (AA-2βG) were gifts from Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan) and Suntory Ltd. (Osaka, Japan), respectively. L-Ascorbic acid (AA) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). D-Erythorbic acid (EA) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The structures of AA and its related compounds are shown in Fig. 1. All purchased reagents were of the highest commercial available purity. All water used was Milli-Q grade.

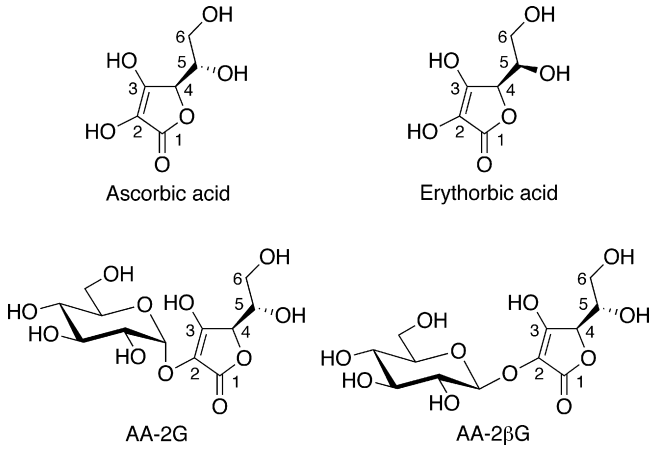

Fig. 1. Chemical structures of ascorbic acid and its related compounds.

2.2. Apparatus and chromatographic conditions

A Shimadzu liquid chromatographic system (Kyoto, Japan) consisting of an SCL-10A system controller, LC-10AD pump, SPD-10AV UV–vis spectrophotometric detector, SIL-10AD auto injector, CTO-6A column oven, DGU-14A degasser and C-R7A Chromatopac was used. The samples (each 10 μl) were injected using a refrigerated autosampler kept at 4 °C. The chromatographic analyses were carried out on an Inertsil Diol (4.6 i.d. × 250 mm, 5 μm, GL Sciences, Tokyo, Japan) kept at 40 °C, using acetonitrile: water with 66.7 mM ammonium acetate (85:15, v/v) as a mobile phase. The flow rate was 0.7 ml/min and the absorbance at 260 nm was monitored.

2.3. Standard preparation and calibration curve

Stock solutions (500 μg/ml) were freshly prepared by dissolving AA, EA, AA-2G and AA-2βG in water. Standard solutions of 1, 2.5, 5, 10, 25 and 50 μg/ml for AA and EA were prepared by diluting the corresponding intermediate stock solution in acetonitrile-66.7 mM ammonium acetate solution (85:15, v/v) containing 50 mg/l of dithiothreitol. Standard solutions of 2.5, 5, 10, 25, 50 and 100 μg/ml for AA-2G and AA-2βG were also prepared. Calibration curves were constructed in the range of 1–50 or 2.5–100 μg/ml to encompass the expected concentrations in measured samples. Calibration curves were constructed by plotting the peak areas of each compound versus concentration and gave the values of slope along with the intercept and correlation coefficient for each calibration curve. Each point was analyzed in triplicate. The calibration curves were used for the quantification of AA, EA, AA-2G and AA-2βG in foods and beverages.

2.4. Method validation

The intra- and inter-day precision and accuracy were evaluated by assaying the quality control (QC) samples with low, medium, and high concentrations (5, 25 and 45 μg/ml for AA and EA; 10, 50 and 90 μg/ml for AA-2G and AA-2βG). QC samples were prepared by diluting the stock solutions and AA-free green tea obtained by heat treatment (final concentration 5%) in acetonitrile-66.7 mM ammonium acetate solution (85:15, v/v) containing 50 mg/l of dithiothreitol. The intra-day variation was determined by assaying six replicates on the same day and inter-day variation was assayed for triplicates on three days. The precision was expressed as the relative standard deviation (R.S.D.). The accuracy was determined by comparing the calculated concentration (obtained from the calibration curve) to the theoretical concentration. Stability of AA, EA, AA-2G and AA-2βG in 20-hold diluted AA-free green tea was assessed with QC samples ($n = 6$) stored in autosampler at 4 °C for 24 and 48 h.

2.5. Sample preparation

Samples were preparated by the relevant procedures as follows. Since AA and EA were very unstable under the preparation conditions, dithiothreitol was added as a stabilizer to the solu-

tion for sample dilution. The final sample solutions were filtered through a PTFE membrane of 0.45 μm in pore size before HPLC analysis.

2.5.1. Tea drinks

Two kinds of PET-bottled teas were used. Chinese jasmine tea and Japanese green tea were purchased from an Internet shop and from a local market, respectively. Jasmine tea was directly diluted five times with acetonitrile–66.7 mM ammonium acetate solution (85:15, v/v) containing 50 mg/l of dithiothreitol, and green tea was diluted 20 times.

2.5.2. Nutritional supplement food

A granular supplement containing AA-2G was a gift from AscorBio Lab. Inc. (Okayama, Japan). An amount of ca. 200 mg of the supplement was weighed, put into a 50-ml volumetric flask and dissolved in an adequate amount of water. The solution was made up to 50 ml with water and was then diluted 40 times with acetonitrile-66.7 mM ammonium acetate solution (85:15, v/v) containing 50 mg/l of dithiothreitol.

2.5.3. Dried fruit

Dried fruits of *Lycium barbarum* were purchased in a local drugstore. They were from Ningxia, which is a well-known production area of the fruit in China. An amount of ca. 1 g of grinded *Lycium* fruit was weighed and put into a 10-ml volumetric flask. After addition of 6 ml of acetonitrile–water (30:70, v/v) containing 200 mg/l of dithiothreitol, the fruit was extracted ultrasonically for 15 min at 0–4 °C, and then the solution was diluted to 10 ml. The extract was centrifuged at 1160 × *g* for 10 min at 4 °C, and the supernatant was diluted 25 times with acetonitrile-66.7 mM ammonium acetate solution (85:15, v/v) containing 50 mg/l of dithiothreitol.

For recovery experiments, an amount of ca. 1 g of grinded *Lycium* fruit, 5.0 mg of AA and EA, and 10.0 mg of AA-2G were put into a 10-ml volumetric flask, and after the addition of 6 ml of 30% acetonitrile–water containing or not containing dithiothreitol (200 mg/l), the fruit was extracted. The subsequent procedure was the same as that described above.

3. Results and discussion

3.1. Choice of HILIC mode

In order to develop a new HPLC method for the simultaneous determination of ascorbic acid (AA), erythorbic acid (EA), 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) and 2-*O*-β-D-glucopyranosyl-L-ascorbic acid (AA-2βG) (Fig. 1), it was necessary to employ a stationary phase with high retention of these high polar compounds. We previously found that RPLC with a CN column had high retention of polar compounds and lower retention of hydrophobic compounds and was able to separate AA, AA-2G and lipophilic AA derivatives [23]. When the possibility of a CN column was explored, it was found that this column was not suitable for the simultaneous determination of AA, EA, AA-2G and AA-2βG (data not shown). Ion-pair RPLC can be used as an alternative method, since measurements of AA and EA in processed meat [8] and measurements of AA and AA-2βG in rat plasma [17] have been carried out using an ODS column with an ion pair reagent. However, we chose a HILIC column rather than an RPLC column for the following reasons: (1) precise quantitation for AA and its related compounds can be expected owing to the rapid elution of hydrophobic sample components and the late elution of polar components, (2) a high organic solvent concentration of mobile phases results in low operating back pressure and ability to run at high flow rates and (3) the high organic solvent content of the mobile phase will lead to a promising LC/MS analysis for identification of unknown AA-related compounds.

3.2. Chromatography optimization

An Inertsil Diol (4.6 i.d. × 250 mm, 5 μm, GL Sciences) was employed for the simultaneous separation of AA, EA, AA-2G and AA-2βG. Preliminary optimization of the separation for the four compounds was carried out under an acidic condition. The suitability of methanol and acetonitrile as organic solvents in the eluent was studied. Acetonitrile was selected over methanol because methanol was too strong an eluent and because the retention and peak shapes of analytes were poor. The optimal mobile phase and its flow rate were found to be acetonitrile–water–formic acid (90:9.5:0.5, v/v) and 0.7 ml/min. Fig. 2 shows a typical chromatogram of a standard mixture of AA, EA, AA-2G and AA-2βG at a detection wavelength of 240 nm. Although this condition was satisfactory for the separation of AA, EA, AA-2G and AA-2βG, AA-2G and AA-2βG, especially AA-2βG, had a small leading peak.

AA-2G has a pK_a value of 3.1 in water [24], and its pK_a value may be slightly higher in a mobile phase containing a high level of acetonitrile. On the other hand, the pK_a value of AA is 4.2 in water [25]. These acidities are caused by the hydroxyl group at the C-3 position of the AA moiety (Fig. 1). AA-2βG and EA are also thought to have chemical properties very simi-

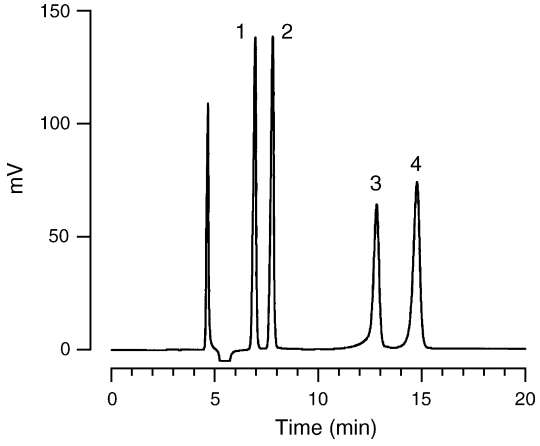

Fig. 2. A typical chromatogram of a standard mixture of AA, EA, AA-2G and AA-2βG using acetonitrile–water–formic acid as a mobile phase. The concentrations of AA, EA, AA-2G and AA-2βG were 25, 25, 50 and 50 μg/ml, respectively. Mobile phase, acetonitrile–water–formic acid (90:9.5:0.5, v/v); flow rate, 0.7 ml/min; detection, UV 240 nm. Peaks: 1. EA; 2. AA; 3. AA-2βG; 4. AA-2G.

lar to those of AA-2G and AA, respectively. The mobile phase might not be sufficient to protonate the acidic hydroxyl group of AA-2G and AA-2βG, since formic acid has a pK_a value of 3.8. Thus, the unsatisfactory peak shape seemed to be caused by imperfect protonation. If the mobile phase is sufficiently acidic to protonate AA-2G and AA-2βG, the AA glucosides would become less hydrophilic and decrease the retention. However, the peak shape and retention time of AA-2G and AA-2βG were not affected, while the peak height and area slightly increased, when acetonitrile–water–trifluoroacetic acid (90:9.9:0.1, v/v) was used as a mobile phase (data not shown). These results indicated that the unsatisfactory peak shape was not caused by imperfect protonation.

Contrary to the above conditions, we sought to separate the deprotonated forms of AA, EA, AA-2G and AA-2βG by using the diol column under neutral conditions. An acetonitrile/salt solution mobile phase was chosen for the chromatography optimization. The isocratic mobile phases used for HILIC ranged from 60 to 90% (v/v) acetonitrile in water with 10 mM ammonium acetate. The detection wavelength of AA, EA, AA-2G and AA-2βG was set at 260 nm, since the maximum absorption wavelength shifts from around 240 nm to around 260 nm under this mobile phase condition. Plots of retention time versus acetonitrile concentration are presented in Fig. 3. An increase in the proportion of acetonitrile in the mobile phase resulted in an increase in all retention times. For AA-2G and AA-2βG, which are more polar than AA and EA, the retention change was dramatic with increasing acetonitrile concentration. The optimal mobile phase and its flow rate were found to be acetonitrile–water with 66.7 mM ammonium acetate (85:15, v/v) and 0.7 ml/min, resulting in a maximum run time of 25 min. Fig. 4 shows a typical chromatogram of a standard mixture of AA, EA, AA-2G and AA-2βG. The retention times were: EA, 11.2 min; AA, 13.0 min; AA-2βG, 16.2 min; and AA-2G, 18.3 min. The analytical methods of polar compounds under HILIC conditions are well studied on TSKgel Amide-80 columns, which consist of carbamoyl groups bonded to a silica gel matrix [26–30]. When a TSKgel Amide-80 (4.6 i.d. × 250 mm, 5 μm, Tosoh) column was used for the determination of AA and its glucosides, the results obtained with the TSKgel Amide-80 column did not exceed the satisfactory results obtained with the Inertsil Diol column (data not shown). These results indicated that this condition was satisfactory for the simultaneous separation of AA, EA, AA-2G and AA-2βG.

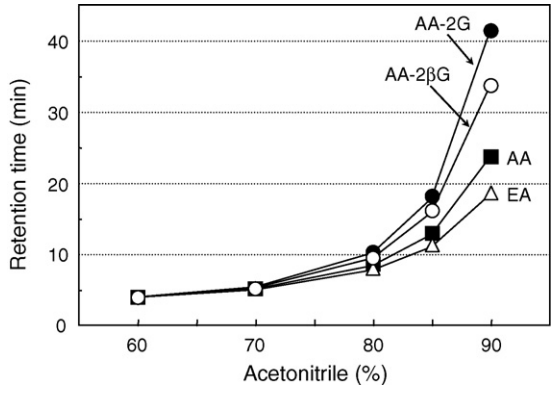

Fig. 3. The effect of acetonitrile content on the retention of AA, EA, AA-2G and AA-2βG. Mobile phase, acetonitrile–water (v/v) containing 10 mM ammonium acetate; flow rate, 0.7 ml/min; detection, UV 260 nm.

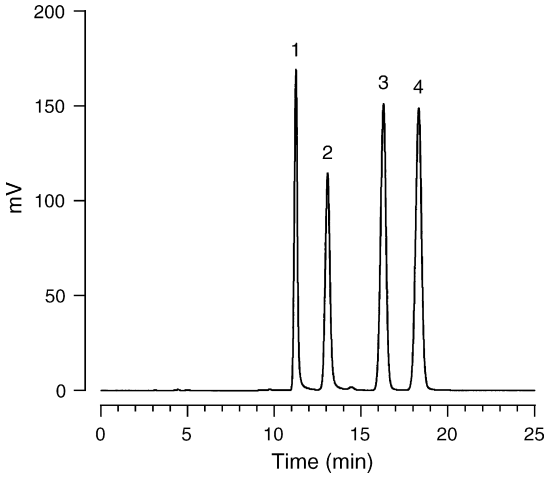

Fig. 4. A typical chromatogram of a standard mixture of AA, EA, AA-2G and AA-2βG using acetonitrile-ammonium acetate as a mobile phase. The concentrations of AA, EA, AA-2G and AA-2βG were 25, 25, 50 and 50 μg/ml, respectively. Mobile phase, acetonitrile-66.7 m*M* ammonium acetate (85:15, v/v); flow rate, 0.7 ml/min; detection, UV 260 nm. Peaks: 1. EA; 2. AA; 3. AA-2βG; 4. AA-2G.

3.3. Linearity and detection limits

Under the optimum experimental conditions, the analytes all showed a good linear relationship, sensitivity and reproducibility. Linear curve fitting was applied to calculate the calibration curves for each standard in the range of 1–50 or 2.5–100 μg/ml. The results are given in Table 1. Excellent linearity was obtained between the peak area (y) and the corresponding concentrations (x) on the standard curve of AA, EA, AA-2G or AA-2βG. The correlation coefficients of the area for AA, EA, AA-2G and AA-2βG were 0.9996, 0.9996, 1.0000 and 1.0000, respectively. The detection limits (signal-to-noise ratio 3:1) for AA, EA, AA-2G and AA-2βG were 0.3 (3), 0.3 (3), 0.03 (0.3) and 0.03 (0.3) μg/ml (ng), respectively. It has been reported that the HILIC mode was applied for the determination of AA and EA [9]. The separation was achieved on an amino column that was pretreated with 0.1 M ammonium monophosphate solution using a mixture of acetonitrile, acetic acid and water (87:2:11, v/v) as an eluant. AA or EA levels as low as 10 ng were detectable at 254 nm. These results indicated that our method is a very sensi-

Table 1
The linearity and detection limits of AA, EA, AA-2G and AA-2βG

Compounds	Regression equation	Correlation coefficient	Detection limit (μg/ml)
AA	$y = 95969x - 85508$	0.9996	0.3
EA	$y = 94109x - 97722$	0.9996	0.3
AA-2G	$y = 71985x - 15290$	1.0000	0.03
AA-2βG	$y = 66521x - 12855$	1.0000	0.03

y: Peak area; x: concentration.

Table 2
Intra- and inter-day precision and accuracy of the method for determination of AA, EA, AA-2G and AA-2βG

Compounds	Added (μg/ml)	Intra-day ($n=6$)		Inter-day ($n=3$)	
		R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)
AA	5	2.8	101.2	1.0	100.2
	25	1.4	105.5	2.1	102.2
	45	1.0	105.8	2.0	103.1
EA	5	2.8	103.0	2.1	103.4
	25	1.3	104.4	1.9	101.6
	45	0.7	104.2	1.9	102.1
AA-2G	10	1.7	98.7	0.6	99.1
	50	0.8	100.6	1.1	100.1
	90	0.7	101.2	1.4	100.0
AA-2βG	10	1.5	103.9	0.5	104.1
	50	0.7	101.8	1.2	101.2
	90	0.5	102.2	1.5	100.8

tive method for detection of AA and its related compounds. The linearity and detection limit of this method were considered to be satisfactory for the simultaneous determination of AA, EA, AA-2G and AA-2βG.

3.4. Precision and accuracy

The intra- and inter-day precision and accuracy of this assay method are shown in Table 2. The precision of the method was calculated as R.S.D. of QC samples at three concentrations in a diluted AA-free green tea. The intra- and inter-day precision (R.S.D.) ranged from 0.5 to 2.8 and 0.5 to 2.1%, respectively. The accuracy was determined by comparing the calculated concentration to the theoretical concentration. The intra-day accuracy was 98.7–105.8% and the inter-day accuracy ranged from 99.1 to 104.1%. These results indicated that the green tea components did not interfere with the quantification and that the present method had a good precision and accuracy for the determination of AA, EA, AA-2G and AA-2βG.

3.5. Sample stability

The stability of AA, EA, AA-2G and AA-2βG solutions stored in autosampler was fully evaluated by analyzing QC samples at three concentrations. The remaining percent of QC samples kept at 4 °C for 24 and 48 h is shown in Table 3. QC samples stored for 24 h showed good stabilities, and the remaining ranged from 99.5 to 102.4%. In QC samples stored for 48 h, AA and EA decreased up to 89.2 and 88.2%, respectively, while AA-2G and AA-2βG did not. The stability of AA and EA tended to decrease with decrease of the concentration. These results indicated that many samples could be processed at one time within 24 h, which would compensate for the shortcoming of relative long analysis time (about 25 min) of this assay.

Table 3
Stability of AA, EA, AA-2G and AA-2βG in autosampler at 4 °C

Compounds	Sample concentration (μg/ml)	% Remaining ± S.D. ($n=6$)	
		24 h	48 h
AA	5	101.6 ± 2.1	89.2 ± 8.0
	25	100.6 ± 1.0	94.3 ± 5.0
	45	101.5 ± 1.1	98.6 ± 2.1
EA	5	102.4 ± 1.4	88.2 ± 11.0
	25	100.1 ± 1.2	95.0 ± 5.2
	45	100.9 ± 0.9	98.5 ± 2.3
AA-2G	10	100.9 ± 0.9	100.6 ± 2.6
	50	99.6 ± 0.9	101.0 ± 1.4
	90	100.0 ± 1.0	101.5 ± 1.3
AA-2βG	10	100.7 ± 0.8	100.7 ± 2.3
	50	99.5 ± 0.7	101.4 ± 1.3
	90	99.9 ± 0.9	101.4 ± 1.3

3.6. Application

All real samples were analyzed according to the optimized conditions. Fig. 5 shows chromatograms of four samples: jasmine tea, green tea, a nutritional supplement food and dried

Table 4
Analytical results of AA, EA, AA-2G and AA-2βG in the real samples

Samples	AA (μg/ml)	EA (μg/ml)	AA-2G (mg/g)	AA-2βG (mg/g)
Jasmine tea	31.9 ± 0.7	23.4 ± 1.4	ND	ND
Green tea	267.5 ± 1.2	ND	ND	ND
Nutritional supplement	ND	ND	260.7 ± 10.6	ND
Dried *Lycium* fruit	ND	ND	ND	7.4 ± 0.5

Results are presented as mean ± S.D. ($n=3$). ND, not detectable.

Table 5
The recovery data of AA, EA and AA-2G in dried *Lycium* fruit

Compounds	Added (mg)	Extraction solvent 1		Extraction solvent 2	
		Analytical result (mg)	Recovery (%)	Analytical result (mg)	Recovery (%)
AA	5.0	4.6 ± 0.3	92	1.9 ± 1.0	38
EA	5.0	4.7 ± 0.3	94	2.2 ± 1.1	44
AA-2G	10.0	9.5 ± 0.2	95	9.7 ± 0.2	97

Results are presented as mean ± S.D. of six separate analyses. Extraction solvent 1 is acetonitrile and water (30/70) containing 200 mg/l of dithiothreitol. Extraction solvent 2 is acetonitrile and water (30/70) not containing dithiothreitol.

L. barbarum fruit. No interference occurred with other sample components, and a good separation for AA, EA, AA-2G and AA-2βG was obtained. Table 4 shows the quantitative results of all samples. AA and EA, and AA as listed in the food label were observed in jasmine tea and green tea, respectively (Fig. 5A and B). Only AA-2G was observed in the nutritional supplement food, and the value was 260.7 mg/g (Fig. 5C and Table 4). The observed values are in good agreement with the content information (250 mg/g) displayed on the food label. From Fig. 5D, AA-2βG was determined as a major component in dried *Lycium* fruit. The content was 7.4 mg/g (Table 4), which is slightly higher than that previously reported (AA-2βG content in the dried fruit, ca. 5 mg/g) [17]. Using this method, AA was not detected in *L. barbarum* extracts, although it has been reported that crude extracts were rich in antioxidant components, such as carotene, ascorbic acid, thiamine, riboflavin, nicotinic acid, zeaxanthin, cryptoxanthin, and coumarin (scopoletin) [31].

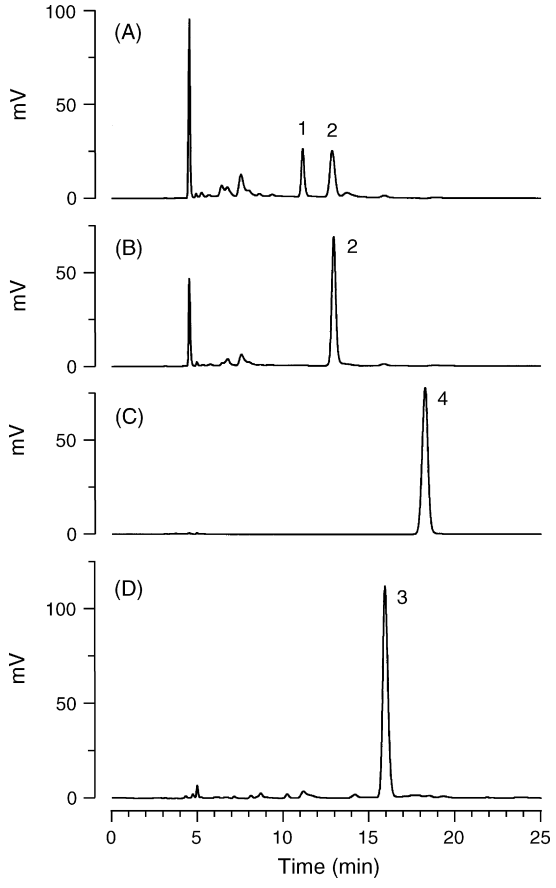

Fig. 5. Chromatograms of four real samples, (A) jasmine tea, (B) green tea, (C) a nutritional supplement food and (D) dried *Lycium barbarum* fruit. Peaks: 1. EA; 2. AA; 3. AA-2βG; 4. AA-2G.

Recovery experiments were carried out to evaluate the accuracy of the method. Suitable amounts of AA, EA and AA-2G were added to grinded *Lycium* fruit, and the mixtures were extracted and analyzed using the proposed procedure. The content and recovery data of dried *Lycium* fruit are shown in Table 5. Recovery was expressed for each component as the mean percentage ratio between the measured amounts and the added amounts. In the case of extraction solvent 1, the observed values were in good agreement with the expected ones. The obtained acceptable recoveries ranged from 92 to 95% ($n = 6$). In the case of extraction solvent 2, however, the observed values of AA and EA were not in agreement with the expected ones, whereas the observed value of AA-2G was in good agreement with the expected one. The contents of AA-2βG in dried *Lycium* fruits were not influenced by either extraction solvent (extraction solvent 1, 7.7 ± 0.2 mg/g; extraction solvent 2, 7.5 ± 0.2 mg/g). The instability of AA and EA in aqueous solutions is well known, while AA-2G and AA-2βG have been found as stable AA derivatives [17,24]. It was thought that AA and EA were decomposed in the extraction process using solvent 2 even at 0–4 °C. Thus, an antioxidant, dithiothreitol, was required for extraction of AA and EA. Therefore, this method can be applied to analysis for unstable AA and EA as well as stable AA derivatives, accompanied by the extraction process. The results indicate that the present method is effective for simultaneous determination of AA and its related compounds in foods and beverages.

4. Conclusions

A new HILIC method was developed for the simultaneous determination of AA, EA, AA-2G and AA-2βG. The HILIC method used an Inertsil Diol (4.6 i.d. × 250 mm, 5 μm, GL Sciences) and a mobile phase consisting of acetonitrile-66.7 mM ammonium acetate solution (85:15, v/v) at a detection wavelength of 260 nm. A HILIC method using an Inertsil CN-3 (4.6 i.d. × 250 mm, 5 μm, GL Sciences) was not tested owing to the satisfactory results obtained with the diol column, while an RPLC method using the CN column was not suitable for the simultaneous determination of AA and its related compounds. In addition, the results obtained with the other HILIC column did not exceed the satisfactory results obtained with the Inertsil Diol column. With our method, good linear relationship and sensitivity were also obtained for standard solutions. This method

was satisfactorily applied to the determination of AA, EA, AA-2G and AA-2βG in a fruit, a food and beverages. The results show that the procedure is simple and sensitive and that it can be employed for the simultaneous determination of AA and its related compounds in foods and beverages.

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