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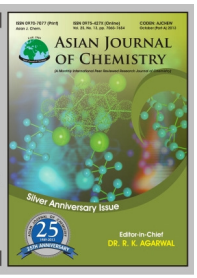

High Performance Liquid Chromatography Analysis of the Functional Components in Jujube Fruit

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In this paper, the functional components in Jujube (*Zizyphus zizyphus*) fruit, including one flavonoid, two pentacyclic triterpenes and two cyclic nucleotides, were analyzed. High performance liquid chromatography and the ultra-high-performance liquid chromatography methods were developed for characterization and quantitation of the five functional components. The established methods could be used to determine subtle differences and explore potential chemical markers for differentiation among jujube fruit from different sources. Distribution of the chemical components among the different jujube fruit samples varied.

Key Words: Jujube, Ultra high performance liquid chromatography, Flavonoid, Cyclic nucleotide, Pentacyclic triterpene.

INTRODUCTION

The jujube (*Zizyphus zizyphus*) is a thorny Rhamnaceous plant that is mainly found in subtropical and tropical regions of Asia and America. In China, its fruit has been used for over 4000 years both as food and in medicine because it has high nutritional value[1] and contains many pharmacologically active components[2]. Phytochemical studies have revealed that the jujube date contains various compounds, including flavonoids[3,4], triterpenic acids[5-7], cyclic nucleotides[8], phenolic acids[9-11], fatty acids[10], amino acids[12], cyclopeptide alkaloids[13] and polysaccharides[14,15].

Currently, high-performance liquid chromatography (HPLC) is commonly used for analysis of functional components in jujube fruit because it has high separation efficiency and is a simple technique. A liquid chromatography method for simultaneous separation and determination of vitamins B-1 (thiamine), B-3 (nicotinic acid), B-6 (pyridoxine) and B-2 (riboflavin) in Chinese jujube (*Ziziphus jujuba* Mill) has been developed and validated[16]. HPLC has been used to analyze various tissues of three jujube varieties for their antioxidant activities and the antioxidants they contain, such as phenolic acids[17]. Evaporative light-scattering detection (ELSD), pulsed amperometric and conductive detection have also been used in combination with HPLC. A reversed-phase HPLC method for the simultaneous characterization and quantitation of 11 triterpenic acids was developed in chloroform extracts of jujube fruit using evaporative light-scattering detection and electrospray ionization mass spectrometry (ESI-MS)[18]. Twelve compounds from methanol extracts have been identified as quercetin, kaempferol and phloretin derivatives by HPLC/ESI-MS analyses[3]. Eight phenolic acids in the pulp, seed and peel of jujube fruit have been separated and quantified by HPLC with electron capture detector[19]. Phenolic compounds, α-tocopherol and β-carotene have been analyzed with an HPLC equipped with a diode array detector[10]. However, a method for analyzing the various functional components simultaneously has not been established.

Compared to HPLC, ultra-high-performance liquid chromatography (UHPLC) is a powerful technique that could be used to separate and characterize the functional components in jujube samples. In earlier studies, an UHPLC was coupled with a photodiode array detector and ESI-MS (UHPLC-DAD-MS) for simultaneous characterization and quantitation of nine nucleosides and nucleobases in 49 jujube samples[8]. Ultra-high-performance liquid chromatography-time-of-flight mass spectrometry method has also been applied to two *Ziziphus* species (*Z. jujuba* and *Z. jujuba* var. *spinosa*)[20].

In the present work, HPLC and UPLC methods were compared for analysis of rutin, ursolic acid, oleanolic acid, adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP) in jujube dates. The functional components in four cultivars of Chinese jujube were also compared.

EXPERIMENTAL

A reference standard of rutin was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Gemany) and cAMP, cGMP

TABLE-1
LINEAR REGRESSION DATA, LIMIT OF DETECTION AND LIMIT OF QUANTIFICATION FOR THE ANALYTES IN JUJUBE SAMPLES (n = 6)

Analytes	Methods	Slope (A ± SD)	Intercept (B ± SD)	R^2	LOD	LOQ
Rutin	HPLC	50779 ± 331	348003 ± 9811	0.9989	0.20	0.70
	UHPLC	34174 ± 122	9349 ± 3756	0.9986	0.01	0.03
Oleanolic acid and Ursolic acid	HPLC	21367 ± 31	149313 ± 9126	0.9891	1.00	5.00
Oleanolic acid	UHPLC	13343 ± 595	776 ± 16	0.9992	0.15	0.40
Ursolic acid	UHPLC	7542 ± 66	17393 ± 1031	0.9995	0.25	0.65
cAMP	HPLC	21757 ± 153	-2753 ± 274	0.9998	0.50	1.20
	UHPLC	16596 ± 231	10806 ± 425	0.9991	0.25	0.70
cGMP	HPLC	7912 ± 13	1471 ± 243	0.9987	0.05	0.20
	UHPLC	5665 ± 73	8454 ± 106	0.9995	0.02	0.05

Notes: $y = Ax + B$, y is the peak area; x is the concentration of the reference compound (μg g^{-1}); SD is the standard deviation; R^2 is the correlation coefficient of the equation; the LOD and LOQ are in μg g^{-1}

and ursolic acid standards were obtained from acros organics (Geel, Belgium). Oleanolic acid was obtained from Tokyo Chemical Industry Co. (Tokyo, Japan).

Methanol of HPLC grade was from Thermo Fisher Scientific (Waltham, MA). Water used in the experiments was purified using a Milli-Q system (Milipore, Billerica, MA). Potassium dihydrogen phosphate (KH_2PO_4) (guaranteed reagent grade) was obtained from Kermel Chemical Reagent Co. Ltd., (Tianjin, China). All working solutions were prepared immediately before analysis.

HPLC was performed on a Shimadzu LC-20 AT, equipped with a Shimadzu SPD-20A UV detector (Shimadzu, Kyoto, Japan). UHPLC analysis was performed with a waters acquity ultra performance LC coupled with a photodiode array detector (Waters, Milford, MA).

Sample preparation for Flavonoid and pentacyclic triterpene analysis: The samples were extracted using an established method[21] with slight modification. Each dry jujube date sample was pulverized into a powder (60 mesh). Ten grams of the pulverized powder was accurately weighed and ultrasonically extracted twice with 100 mL of ethanol for 45 min in a conical flask at 75 ºC each time. The combined extract was filtered through analytical filter paper and then evaporated to dryness in a rotary evaporator. The residue was dissolved with methanol in a 50 mL volumetric flask and filtered through a 0.22 mm membrane filter.

Sample preparation for cyclic nucleotide analysis: The samples were extracted using an established method[19]. The dry jujube date sample was pulverized into powder (60 mesh). Ten grams of the pulverized powder was accurately weighed and ultrasonically extracted with 100 mL of deionized water for 1 h in a conical flask at 75 ºC. The extract was then centrifuged at 1500 rpm for 15 min. This step was repeated and the combined extract was evaporated to dryness in a rotary evaporator. The residue was dissolved with deionized water in a 50 mL volumetric flask and filtered through a 0.22 mm membrane filter.

HPLC and UHPLC conditions for flavonoid and pentacyclic triterpene analysis: HPLC and UHPLC were performed using reversed-phase symmetry C_{18} (250 mm × 4.6 mm I.D., 5 μm, waters) and acquity UPLC BEH C_{18} (100 mm × 2.1 mm I.D., 1.7 μm, waters) columns, respectively. In each case, the column temperature was maintained at 30 ºC and the detection wavelength was set at 210 nm. For HPLC the mobile phase consisted of solvent A (0.03 % aqueous phosphoric acid, v/v) and B (methanol) with the following linear gradient elution: 0-4 min, 40-20 % A; 4-5 min, 20-1% A; 5-12 min, 1 % A; 12-13 min, 1-40 % A; and 13-16 min, 40 % A. The mobile phase flow rate was 0.7 mL min^{-1}. For UHPLC the mobile phase was 0.03 % aqueous phosphoric acid (v/v) and methanol (11:89, v/v) and the mobile phase flow rate was 0.1 mL min^{-1}.

HPLC and UHPLC conditions for cyclic nucleotide analysis: HPLC and UHPLC were performed using reversed-phase symmetry C_{18} (250 mm × 4.6 mm I.D., 5 μm, waters) and acquity UPLC BEH C_{18} (100 mm × 2.1 mm I.D., 1.7 μm, waters) columns, respectively. In both cases, the column temperature was maintained at 30 ºC, the wavelength was set at 254 nm and the mobile phase was 20 mmol L^{-1} KH_2PO_4 and methanol (90:10, v/v). The flow rate of the mobile phase was 1.0 mL min^{-1} for HPLC and 0.25 mL min^{-1} for UHPLC.

RESULTS AND DISCUSSION

Linearity, limit of detection and limit of quantification: Six-point calibration curves (5, 10, 20, 30, 50 and 100 μg g^{-1}) were constructed. All analyte responses were linear over the concentration range investigated. The limit of detection and limit of quantification were determined using injections of dilute standard solutions until the concentrations for the analytes reached three or 10 times the standard deviation of the apparent concentration of blank samples, respectively (Table-1).

Repeatability: Method validation was performed after optimizing the factors affecting the analysis procedure. The repeatability of the method was investigated with six replicate analyses of the standard analytes at two concentration levels (10 and 20 μg g^{-1}). The recoveries were all between 73.1 % and 92.6 % (Table-2), which indicates that the extraction method is repeatable.

Application to real samples: The methods were used to analyze four cultivars of Chinese jujube. These samples showed obvious differences in their compositions (Table-3).

Comparison of the HPLC methods and the UHPLC methods: HPLC and UHPLC methods were compared for flavonoid, pentacyclic triterpene and cyclic nucleotide analysis. Both techniques were rapid, simple, did not need much solution and showed good repeatability and recovery. However, UHPLC had better accuracy, precision, limit of detection and analytical

TABLE-2
COMPARISON OF THE RECOVERIES OF THE DIFFERENT ANALYTES WITH THE PROPOSED METHODS (n = 6)

Analytes	Methods	10 μg g^{-1} mean recovery (%) ± SD (%)	20 μg g^{-1} mean recovery (%) ± SD (%)
Rutin	HPLC	87.8 ± 6.5	85.4 ± 1.0
	UHPLC	82.2 ± 3.4	92.6 ± 1.5
Oleanolic acid and ursolic acid	HPLC	73.7 ± 7.9	81.9 ± 6.4
Oleanolic acid	UHPLC	75.3 ± 6.0	79.9 ± 1.4
Ursolic acid	UHPLC	73.6 ± 2.8	76.5 ± 4.4
cAMP	HPLC	89.2 ± 7.5	73.1 ± 2.7
	UHPLC	74.1 ± 3.8	78.3 ± 8.8
cGMP	HPLC	74.54 ± 4.7	84.7 ± 2.3
	UHPLC	82.5 ± 5.7	90.4 ± 4.8

efficiency than HPLC. UHPLC had better resolution than HPLC (Fig. 1). The resolution of oleanolic acid and ursolic acid for UHPLC was > 1.5 and with HPLC it was 0.7.

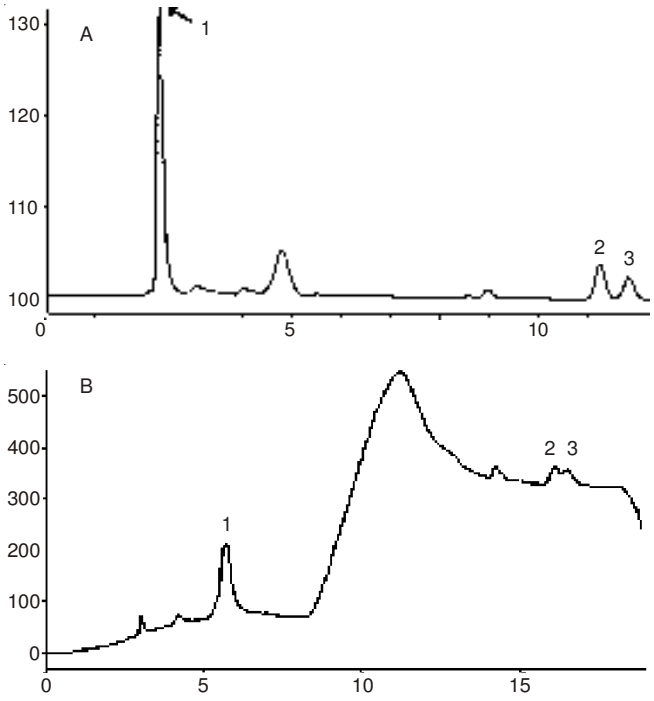

Fig. 1. Chromatograms of the flavonoid and pentacyclic triterpene standards; A: UHPLC chromatograms of the standards. B: HPLC chromatograms of the standards; peaks: 1, rutin; 2, oleanolic acid; 3, ursolic acid

Pentacyclic triterpene content: Miao[22] studied 79 cultivars of Chinese jujube and found oleanolic acid contents of 23-388 μg g^{-1} and ursolic acid contents of 7-420 μg g^{-1}. The four cultivars of Chinese jujube were not as rich in oleanolic acid and the ursolic acid (Table-3).

In an earlier study[18], HPLC simultaneous characterization and quantitation of 11 triterpenic acids showed the the oleanonic acid content was 54.4 μg g^{-1} and that of ursolic acid was 22.3 μg g^{-1}. In our research, the oleanonic acid content range was 48.0-63.2 μg g^{-1} and the ursolic acid range was 83.8-150.5 μg g^{-1}.

Cyclic nucleotide content: The HPLC conditions in the present study were similar to those in an earlier report[23]. Compared to the results of that report, the cAMP contents in the Junzao and Hupingzao samples in the present study were much higher. Using an UHPLC-DAD-MS method[8] characterized and quantified nine nucleosides and nucleobases in 49 jujube dates samples. The cAMP content range was 0-413.47 μg g^{-1} and the cGMP content range was 5.88-159.02 μg g^{-1}. By comparison, present results for the Junzao and Hupingzao sample contents were in the middle of these ranges.

TABLE-3
ANALYSIS OF FOUR CULTIVARS OF CHINESE JUJUBE (n = 3)

Analytes	*Zizyphus jujuba*			
	Junzao	Hupingzao	Banzao	Muzao
Rutin	938.2 ± 25.9^{c}	1042.8 ± 26.0^{b}	729.1 ± 23.5^{d}	1529.0 ± 22.1^{a}
Oleanolic acid	57.1 ± 16.0^{a}	60.2 ± 10.2^{a}	48.0 ± 5.2^{b}	63.2 ± 6.7^{a}
Ursolic acid	83.8 ± 45.4^{d}	106.1 ± 10.6^{c}	127.1 ± 10.4^{b}	150.5 ± 11.8^{a}
cAMP	53.2 ± 4.6^{a}	51.7 ± 0.2^{a}	7.7 ± 0.8^{b}	8.8 ± 2.3^{b}
cGMP	35.0 ± 4.6^{a}	35.3 ± 2.2^{a}	1.8 ± 0.0^{c}	9.2 ± 0.3^{b}

Notes: Each value is expressed as the mean ± standard deviation (μg g^{-1}). Different letters within a row indicate that the means are significantly different ($p < 0.05$)

Proximate comparison of the samples: The functional components of four cultivars of Chinese jujube were compared (Table-3) by multivariate analysis. The rutin content range of the different Chinese jujube samples was 729.1-1529.0 μg g^{-1} and the samples exhibited significant differences ($p < 0.05$). No significant differences were observed for the oleanolic acid contents among the four cultivars of Chinese jujube, but ursolic acid exhibited significant differences in each cultivar ($p < 0.05$). The Muzao sample had the highest rutin and ursolic acid contents. The ursolic acid contents were higher than the oleanolic acid contents in all studied samples. The cAMP content range was 7.7-53.2 μg g^{-1} and the cGMP content range was 1.8-35.3 μg g^{-1}.

Conclusion

UHPLC and HPLC are reliable and simple analytical methods for analysis of the functional components of Jujube dates. HPLC and UHPLC data were compared and correlation plots showed excellent agreement the between values obtained for flavonoids, pentacyclic triterpenes and cyclic nucleotides. UHPLC provided better precision and efficiency than HPLC.

Four cultivars of Chinese jujube had clearly differently functional compositions and jujube fruit were a good source of flavonoids, pentacyclic triterpenes and cyclic nucleotides.

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