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High-performance liquid chromatographic determination of kaempferol glycosides in Cinnamomum osmophloeum leaves

Yerra Koteswara Rao[a], Madamanchi Geethangili[a], Hsiao-Sung Chan[a], Wen-Shi Wu[b] and Yew-Min Tzeng[a] *

[a]*Institute of Biochemical Sciences and Technology, Chaoyang University of Technology, Wufeng, Taiwan, ROC*
[b]*Department of Horticulture and Biotechnology, Chinese Culture University, Taipei, Taiwan, ROC*

Abstract: Traditional Chinese medicinal plant *Cinnamomum osmophloeum* Kaneh (Lauraceae) has been used as spices and condiments. A simple and accurate reversed-phase high-performance liquid chromatographic (RP-HPLC) separation method was developed for the determination of two bioactive flavonol glycosides, namely kaempferol-3,7-O-α-L-dirhamnoside (kaempferitrin, **1**), and kaempferol 3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-7-O-α-L-rhamnopyranoside (**2**), in leaves of *C. osmophloeum*. Separation of the two compounds was achieved with a Hypersil BDS C18 column by gradient elution using acetonitrile-water (30:70, v/v) containing 0.1% trifluroacetic acid as a mobile phase. The flow rate and detection wavelength was set at 0.8 ml/min and 265 nm, respectively. Calibration curves were found to be linear within the range of 5–100 μg/mL. The contents of two flavonoids in *C. osmophloeum* leaves were successfully determined with retention times of 14.8 and 15.5 min. The recovery of the two flavonoids was 98.0 and 99.3, respectively. The results indicated that the developed HPLC assay could be readily utilized as a quality control method for *C. osmophloeum* and its related traditional Chinese medicinal preparations.

Keywords: Cinnamomum osmophloeum; High-performance liquid chromatography; Kaempferol glycosides.

1. Introduction

The genus *Cinnamomum* comprises about 250 species that are distributed in Asia and Australia [1]. Owing to the unique ecosystem, there are many valuable tree species endemic to Taiwan. *Cinnamomum osmophloeum* Kaneh. (Lauraceae), named as "Indigenous cinnamon tree", is one of the endemic hardwood species that grows in Taiwan at an elevation between 400-1500 m [2]. In Taiwan *C. osmophloeum* leaves and bark are used traditionally for treating diabetics and inflammatory diseases, in addition to spices and condiments [3]. The chemical constituents of leaf essential oils were different for various *C. osmophloeum* clones found in different regions of Taiwan [4].

* Corresponding author; e-mail: ymtzeng@cyut.edu.tw
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Several researchers have studied and analyzed composition of *C. osmophloeum* leaves, because the chemical constituents of its leaf essential oils are similar to those of *Cinnamomum cassia* bark oil commercially known as “cinnamon oil” which is commonly used in the food and beverage industries because of its special aroma [3,5]. In addition to its useful essential oils, the main bioactive secondary metabolites from the leaves of *C. osmophloeum* are flavonol glycosides [6].

Epidemiological studies suggested that flavonoids have various biological activities such as to decrease the risk of cardiovascular diseases [7] and cancer [8] and so on. Importantly, the analytes in this study, kaempferol -3,7-O-α-L-dirhamnoside (kaempferitrin, **1**), and kaempferol 3-*O*-*β*-D-glucopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-7-*O*-*α*-L-rhamnopyranoside (**2**) (Figure 1) are reported to have anti-inflammatory activity [6]. In addition, compound **1** was also reported to have significant biological activities such as, insulinomimetic [9], hypoglycemic and antioxidant [10], and protective effects on the renal cellular membrane [11]. Furthermore, the authors reported that to it was also reported that compounds **1** and **2** have insulin-mimetic activities on regulation of adiponectin secretion, tyrosine phosphorylation of insulin receptor-*β* and glucose transporter 4 in mouse 3T3-L1 adipocytesare [12, 13]. With the increasing applications of compounds **1** and **2** in medicinal purpose, it is necessary to establish an analytical method for their determination in biological samples.

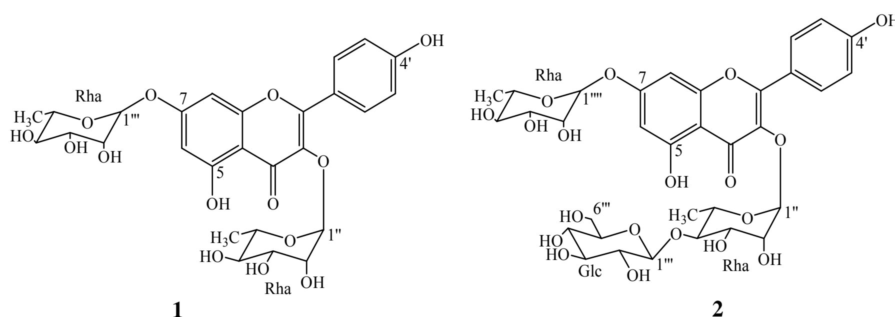

Figure 1. Chemical structures of kaempferitrin (1) and kaempferol 3-*O*-*β*-D-glucopyranosyl-(1→4)-*α*-L-rhamnopyranosyl-7-*O*-*α*-L-rhamnopyranoside (2).

High-performance liquid chromatographic (HPLC) are widely used analytical method for the analyses and quantification of bioactive compounds (flavonoids) in plant extracts [14]. To our knowledge, no other work on the qualitative and quantitative analysis of kaempferol glycosides from *C. osmophloeum* leaves has been previously reported. Therefore, for the first time, this paper presents an HPLC method for the quantitative determination of kaempferol glycosides in the crude extract of *C. osmophloeum* leaves.

2. Materials and Methods

2.1. Plant material and chemicals

The leaves of *C. osmophloeum* were collected in September 2007 from the Yuli, Hualien County in Eastern Taiwan, and a botanically identified voucher specimen (YMT- 07-05) was deposited in the Herbarium of the Institute of Biotechnology, Chaoyang University of Technology, Taiwan.

HPLC-grade methanol and acetonitrile were purchased from Tedia Chemical Co., USA. Deionized water was prepared by a Milli-Q water purification system (Millipore, MA). All the solutions were filtered through 0.45 µm membranes (Schleicher & Schuell, Germany) and degassed by an ultrasonic bath before use.

2.2. Isolation and characterization of compounds 1 and 2

Powdered dry leaves of *C. osmophloeum* (500 g) were extracted with methanol: water (8:2×2L) using soxhlet under reflux. The extract was filtered, concentrated under reduced pressure. The concentrated extract (23 g) was dissolved in water and then extracted with *n*-hexane followed by *n*-butanol successively. The concentrated *n*-butanol fraction (14 g) was chromatographed on silica gel column, and eluted with chloroform–methanol of increasing polarity to obtain two fractions A–1 and A–2. Fraction A–1 was subject to dry-flash chromatography using chloroform–methanol of increasing polarity to give kaempferol-3,7-O-α-L-dirhamnoside (kaempferitrin, **1,** 156 mg). Fraction A–2 was repeatedly chromatographed on silica gel column with chloroform–methanol of increasing polarity to give kaempferol 3-O-β-D-glucopyrasyl-(1→4)-α-L-rhamnopyranosyl-7-O-α-L-rhamnopyranoside (**2**) (19 mg). The spectral data of compounds **1** and **2** were presented in Table 1 and were consistent with the published data [6].

2.3. Sample preparation for HPLC analysis

The *C. osmophloeum* leaves (1 g) was extracted with 50 mL of methanol: water (8:2) using soxhlet under reflux. The filtrate was evaporated to dryness and the residue was dissolved in 1 mL of methanol. The solution was filtered through a 0.45 µm micropore membrane prior to use. A diluted volume of 5 µL was injected into the HPLC instrument for analysis.

2.4. HPLC conditions

An analytical HPLC system consisted of a Hitachi (Tokyo, Japan) L-7100 pump; a 20 µL fixed loop, and a Model L-7400 diode array detector (DAD) was used. Separation was carried out with a Hypersil BDS C18 column (4.6mm×100 mm, 3 µm particle size, Hewlett Packard, Palo Alto, CA). The mobile phase consisted of acetonitrile (solvent A) and water containing 0.1% trifluroacetic acid (solvent B). A linear gradient program was used as follows: 10% A in the first 0 min, linearly gradient to 30% A over 20 min, then hold for 10 min. The mobile phase flow rate was 0.8 mL/min, and the detector was monitored at 265 nm. All the chromatographic operations were carried out at ambient temperature (25 ºC). For calibration curves, appropriate volumes of the standard stock solutions (1 mg/mL) were diluted with methanol, and six concentration levels (5, 10, 20, 40, 80 and 100 µg/mL) were analyzed. For quantification, peak areas were correlated with the concentrations according to the calibration curve. All data are expressed as mean ± standard deviation of triplicate independent experiments (n = 3).

2.5. Precision, accuracy and recovery studies

The intra-day precision of the assays performed in replicate (n = 5) were tested by using three concentrations of the individual flavonol glycosides, namely, 20, 40 and 60 µg/mL. The inter-day precision of the assays was estimated from the results of three replicate assays on 5 different days within 1 week. The precision was evaluated by the relative standard deviation (R.S.D.). Recovery studies were performed by adding suitable amounts of standard stock solutions to crude extract. The samples were treated as described

above. Determinations for recovery studies were performed in duplicate.

Table 1. ^{1}H and ^{13}C NMR spectral data for compounds **1** and **2**[a]

Position	^{1}H NMR ($J_{H,H}$ in Hz)		^{13}C NMR	
	1	**2**	**1**	**2**
2			157.8	157.8
3			134.5	134.7
4			178.0	177.9
5			161.0	160.9
6	6.45 (*d*, 2.4)	6.46 (*d*, 1.8)	99.5	99.5
7			161.7	161.7
8	6.79 (*d*, 2.4)	6.79 (*d*, 1.8)	94.6	94.6
9			156.1	156.1
10			105.8	105.8
1′			120.4	120.3
2′, 6′	7.79 (*d*, 8.4)	7.80 (*d*, 9)	130.8	130.7
3′, 5′	6.92 (*d*, 8.4)	6.94 (*d*, 9)	115.5	115.5
4′			160.2	160.3
5-OH	12.60 (*s*)	12.58 (*s*)		
4′-OH	10.26 (*s*)	10.31 (*s*)		
1″	5.30 (*d*, 1.8)	5.21 (*d*, 1.2)	101.9	102.0
2″	3.98 (*s*)	4.05 (*s*)	70.1	69.8
3″	3.63 (*m*)	3.27-3.45 (*m*)	70.3	69.8
4″	3.40-3.49 (*m*)	3.27-3.45 (*m*)	71.1	81.9
5″	3.40-3.49 (*m*)	3.27-3.45 (*m*)	70.7	69.0
6″	0.80 (*d*, 6.0)	0.92 (*d*, 6)	17.5	17.4
1‴	5.55 (*d*, 1.8)	4.30 (*d*, 7.8)	98.4	104.7
2‴	3.84 (*s*)	3.03 (*dd*, 7.8, 8.4)	70.1	74.5
3‴	3.10-3.17 (*m*)	2.98 (*t*, 8.4)	70.2	77.0
4‴	3.10-3.17 (*m*)	3.07 (*t*,9.6)	71.6	70.3
5‴	3.31 (*m*)	3.15 (*ddd*, 3.0, 6.0, 9.6)	69.8	76.6
6‴ (A)	1.12 (*d*, 6.0)	3.75 (*dd*, 3.0, 11.4)	18.0	60.9
(B)		3.62 (*dd*, 6.0, 11.4)		
1⁗		5.55 (*d*, 1.2)		98.4
2⁗		3.84 (*s*)		70.1
3⁗		3.27-3.45 (*m*)		70.2
4⁗		3.27-3.45 (*m*)		71.6
5⁗		3.27-3.45 (*m*)		69.8
6⁗		1.12 (*d*, 6)		18.0

[a] All NMR data were recorded using DMSO-*d*6; δ values in ppm

3. Results and Discussion

3.1. Optimization of chromatographic conditions

The chromatographic conditions were optimized to obtain chromatograms with a good resolution of the target compounds within a short analysis time. Different types of chromatographic columns such as Mightysil RP-18 column, Hypersil BDS C18 column and Micra NPS ODS-1 column were tested. The kaempferol glycosides in *C. osmophloeum* leaves showed different retention behaviors on these columns. The analysis time did not vary significantly on three columns, while the resolution of Hypersil BDS C18 column was better than the rest two. Thus, Hypersil BDS C18 column was used for analysis.

Methanol and acetonitrile have been widely used chromatographic mobile phase in the investigations of flavonoids [15]. Due to the similar structure and biological properties of flavonol glycosides, it is difficult in the separation and quantification of them, by adding 1% THF in solvent A, the problems appeared before were solved successfully, according to the result of Wang and Huang [16]. In this experiment, several mobile phases, including methanol–water and acetonitrile–water in combination with acetic acid or sodium acetate, were tested.

Initially, when a mobile phase comprising 30% of methanol and 0.1% of trifluroacetic acid in water at pH 3.0 was used, a long retention time of more than 25 min was required for separation of the target compounds. We found that by increasing the proportion of methanol in the mobile phase properly, not only the analyte peaks appeared ahead of time but also the shape of the peaks appeared much more symmetrically. Varying pH of the mobile phase from 2.5 to 5.0 has no effect on the retention times as well as the peak shape of the target compounds. Therefore, for the separation of two target compounds from the matrix, gradient elution of acetonitrile and water containing 0.1% acetic acid at pH of 3.0 (30:70) was chosen as the eluting solvent system. Since this solvent system provides more successful separation and is friendly to the column [17]. Further, increasing the acetonitrile content in the mobile phase to 50% resulted in poor resolution of the two flavonoid peaks. Based on the UV absorption maxima of most flavonoids obtained by HPLC-DAD, the monitoring wavelength chosen was 265 nm [18]. These preliminary experiments also showed that the separation could be improved with column temperature at 25 ºC at a flow rate of 0.8 mL/min.

3.2. Comparison of different solvents

Ethanol, methanol, ethyl acetate and water were used to investigate the extraction effect of two flavonoids comparing with water extraction. It was found that methanol/water 80% (v/v) gave the highest extraction yield for compounds **1** and **2**. The effects of extraction time on the content of compounds **1** and **2** were investigated using 80% (v/v) methanol/water as the solvent. It was found that 30 min of reflux was sufficient to extract the analytes.

3.3. Validation of the method

Typical chromatogram is shown in Figure 2. The retention times of standard compounds **1** and **2** were 14.5 and 15.9 min, respectively (Figure 2A). Peak area (y) of the analytes **1** and **2** was measured and plotted against the concentration (x) of each compound. In the range of 5–100 μg/mL, good correlation of linearity has been achieved. The regression curves and correlation coefficients were $y = 10.285x - 0.477$ (n=6; $R^2 = 0.9993$) for compound **1**, $y = 2.1794x + 1.4772$ (n=6; $R^2 = 0.9976$) for compound **2**. The limit of detection, defined as the lowest sample concentration which can be detected (signal-to-noise ratio = 3), was 1.0 μg for compounds **1** and **2**, and the limit of quantification, defined as the lowest sample concentration which can be quantitatively determined with suitable precision and

accuracy (signal-to-noise ratio > 10), was 5 μg (R.S.D. < 5%) for each kaempferol glycoside. The precision of the analytical method was determined by at least triplicate applications of each sample. One standard solution was analyzed for six times, consecutively, using the analytical method above. The relative standard deviation of peak areas was 1.8% and 1.1% for compounds **1** and **2**, respectively. The study of stability was performed on 2 consecutive days (n = 10) indicating a relative standard deviation of 2.3% and 2.6% for compounds **1** and **2**, respectively. A study of the repeatability showed relative standard deviations of 1.7% and 0.8% for compounds **1** and **2**, respectively, and those for reproducibility between days (n = 10, 3 consecutive days) from 1.2% and 3.4%.

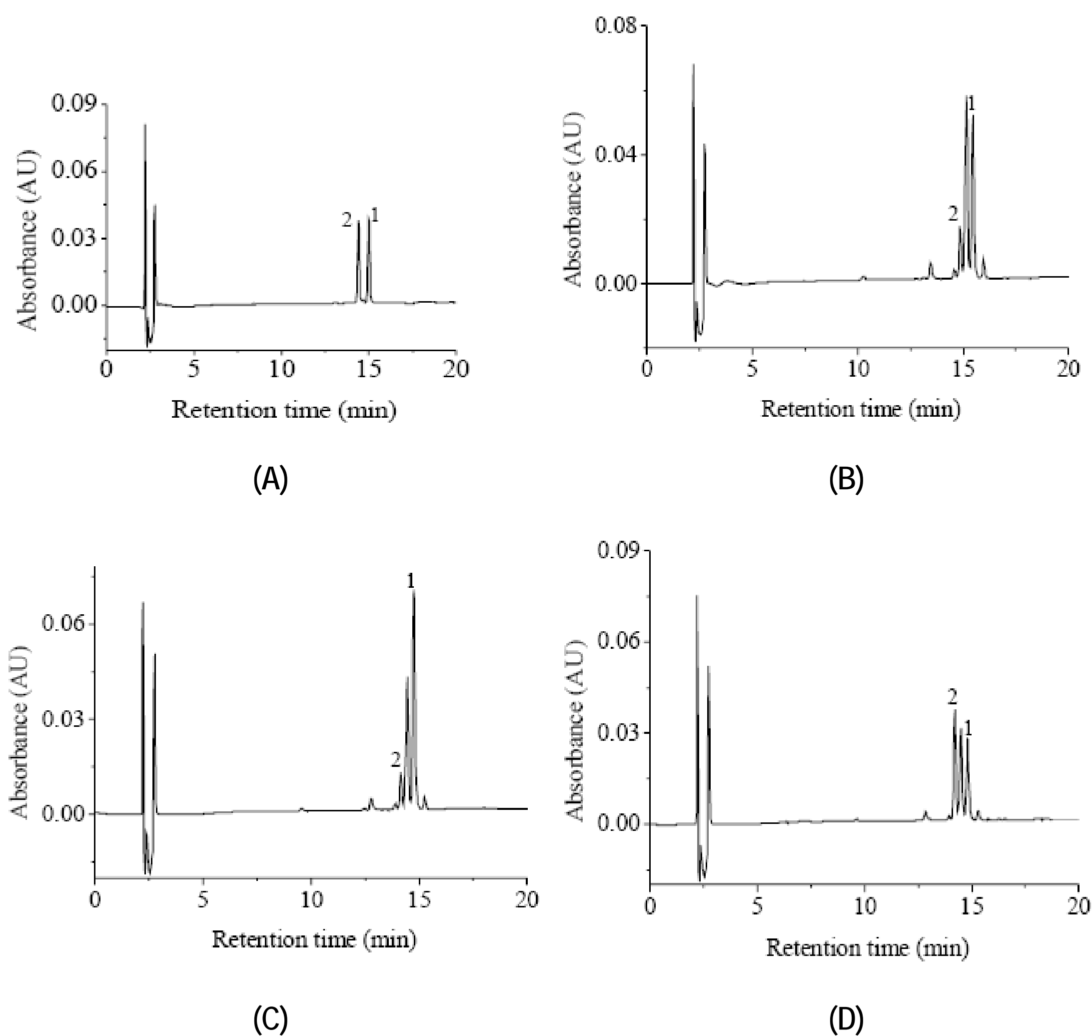

Figure 2. Representative HPLC profiles: (A) standard mixture of compounds 1 and 2; (B) Diluted extract of *C. osmophloeum* leaves; (C) and (D) extracted sample spiked with compound 1 and 2, respectively (spiked concentration 20 μg/mL of standard compound before extraction).

3.4. Sample analysis and recovery

Six samples in different age leaf samples from different age trees of *C. osmophloeum* leaves were extracted, following the

procedure above. The volume of 5 μL of the filtered solution was injected directly and separated under the optimum condition mentioned earlier. Each sample was determined in triplicate. Peaks in the chromatograms were identified by comparing the retention times and on-line UV spectra with those of the standards. Retention time for compounds 1 and 2 were 14.8 and 15.5 min, respectively (see Figure 2B). The content of two kaempferol glycosides in 6 samples of *C. osmophloeum* leaves was determined by the corresponding regression equation and was given in Table 2. It shows that the content of compounds 1 and 2 ranged from 168.2 to 260.8 and 17.5 to 32.7 μg/g, respectively. Comparative determination of compounds 1 and 2 showed that significantly higher yield was obtained in two years leaves of three years tree (Table 2). The recovery was calculated by comparing peak area ratios of the samples after extraction with peak area ratios of standard solutions at the same concentration. The identity of the peak of compounds 1 and 2 was confirmed by the proportional increase of the chromatographic peak caused by the addition of standard to the sample (Figure 2C & D). The average recoveries of compounds 1 and 2 are listed in Table 3. High recovery suggested that there was negligible loss of those two kaempferol glycosides during the extraction process

Sample number[b]	Compound **1**	Compound **2**
3–1	168.2 ± 2.6	17.5 ± 1.1
3–2	260.8 ± 5.1	32.7 ± 1.6
4–4	250.2 ± 4.6	29.6 ± 3.3
5–1	194.9 ± 2.1	19.3 ± 0.7
6–1	204.2 ± 8.7	22.4 ± 1.8
6–2	253.4 ± 6.2	31.2 ± 3.7

Table 2. Contents of compounds 1 and 2 in different samples (μg/g) [a]

[a] Values are means ± standard deviations of triplicate determinations

[b] The numbers 3–1, 3–2, 4–4, 5–1, 6–1, 6–2 represents, the first digit represents the age of the tree and the second digit represents the age of the leaves, respectively.

Compound	Added (μg)	Found* (μg)	Recovered*(μg)	Recovery* (%)	R.S.D. (%)
1	75	260.8 ± 5.07	329.2 ± 3.26	98.0	1.8
2	50	32.7 ± 1.60	82.1 ± 0.86	99.3	2.6

Table 3. Recoveries of two compounds (*n*=6)

"Found*" means the flavonol glycosides of *C. osmophloeum* actual content.

"Recovered*" means the content of the same in final. Recovery* (%) = [(found−actual)/added] × 100%.

4. Conclusion

The present study developed a new method for determination of two kaempferol glycosides in of *C. osmophloeum* leaves. The results showed that this new method was simple, practical, and feasible with high precision, sensitivity and repeatability. On the basis of the developed method, it was investigated for the first time, to our

knowledge, about the evolution of kaempferol glycosides in *C. osmophloeum* leaves with a short analysis time (<16 min).

Acknowledgements

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