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# SIMULTANEOUS HPLC–DAD ANALYSIS OF FOUR METHYLATED FLAVONOLS IN *AMOMUM KOENIGII*

*C. F. Qiao*, *Q. B. Han*, *J. Z. Song*, *H. Yiu*, and *H. X. Xu*[*]

Chinese Medicine Laboratory, Hong Kong Jockey Club Institute of Chinese Medicine, Hong Kong, China

## SUMMARY

Four bioactive methylated flavonols isolated from *Amomum koenigii* J.F. Gmelin, 3,7-dihydroxy-5,3′,4′-trimethoxyflavone (**1**), 5-hydroxy-3,7,4′-trimethoxyflavone (**2**), 3,7-dihydroxy-5,4′-dimethoxyflavone (**3**), and 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (retusin, **4**), have been analyzed by HPLC–DAD on a 4.6 mm × 150 mm, 5-μm particle, $C_8$ column with an acetonitrile–aqueous acetic acid gradient as mobile phase. UV detection was at 360 nm. The method was validated in detail. The results indicated that the total amounts of these four constituents in the seeds were 0.776–0.829%, higher than in the pericarp. Retusin was the major constituent with a maximum content of approximately 0.50% in the seeds. This method is useful for quality control of the crude drug.

## INTRODUCTION

Many species of Zingiberaceae, including ginger (*Zingiber officinale* Roscoe), turmeric (*Curcuma longa* L.), and cardamom (*Elettaria cardamomum* Maton), have been used for centuries as foods, spices, and perfumes, and in traditional Chinese, Japanese, and Indian medicines [1]. *Amomum* Roxb., an important genus in Zingiberaceae with more than 150 species, is widely distributed in the tropics and subtropics of Asia, Australia, and the Pacific islands [2]. Many *Amomum* fruits, for example *A. villosum* Lour. (Sha-Ren) and *A. kravanh* Pierre ex Gagnep. (Bai-Dou-Kou), are commonly used in traditional Chinese medicine as aromatic stomachic tonics [3]. *Amomum koenigii* J.F. Gmelin, distributed in the Guangxi and Yunnan provinces of China and in Thailand and India is not only an edible vegetable but also used in folk remedies to treat stomach ache [2,4]. In some areas, the fruits of this plant are used as substitutes of Fructus Tsaoko (*Amomum*

*tsao-ko* Crev. et Lem.), a popular natural spice and traditional medicine [5]. Previous phytochemical investigation identified eleven methylated flavonols, including 3,7-dihydroxy-5,3′,4′-trimethoxyflavone (**1**), 5-hydroxy-3,7,4′-trimethoxyflavone (**2**), 3,7-dihydroxy-5,4′-dimethoxyflavone (**3**), and 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (retusin, **4**), and two eicosenones in the fruits of *A. koenigii* [6]. Many pharmacological studies have shown that compounds **1**–**4** have diverse biological activity, for example antifungal [7,8], antibacterial [9], ATP synthesis inhibitory [10], cytotoxic [11,12], anti-plasmodial [13], hepatoprotective [14], and anti-emetic [15] effects.

Because these four bioactive methylated flavonols were obtained in high yield from *A. koenigii* in our previous studies, we were interested in establishing a reversed-phase high-performance liquid chromatographic (RP-HPLC) method for quantification of the amounts present in this plant, to provide evidence that these potential resources should be investigated further. In this study, a new, validated, simple, and sensitive RP-HPLC method with diode-array detection (DAD) has been developed for simultaneous determination of **1**–**4** in the fruits of *A. koenigii*.

## EXPERIMENTAL

### Chemicals, Reagents, and Plant Materials

Deionized water was prepared and distilled twice using a Milli-Q SP reagent water system. HPLC-grade acetonitrile and acetic acid were products of International Lab, USA. Chloroform, ethyl acetate, acetone, and methanol were products of VWR, UK. Reference standards of compounds **1**–**4** were isolated from the fruits of *A. koenigii* in our laboratories; their structures were confirmed by MS, and $^{1}H$ and $^{13}C$ NMR, and by comparison with literature data [6]. The purity of the compounds was >98% (determined by normalization of the peak areas detected by HPLC with ELSD). The structures of the compounds are shown in Fig. 1. Samples of *A. koenigii* and *A. tsao-ko* were collected from Guangxi and Yunnan Province, China. Voucher specimens were authenticated by Dr Chun-feng Qiao and deposited in the laboratory of the Hong Kong Jockey Club Institute of Chinese Medicine.

### Sample Preparation

Samples were pulverized and the powder was screened through

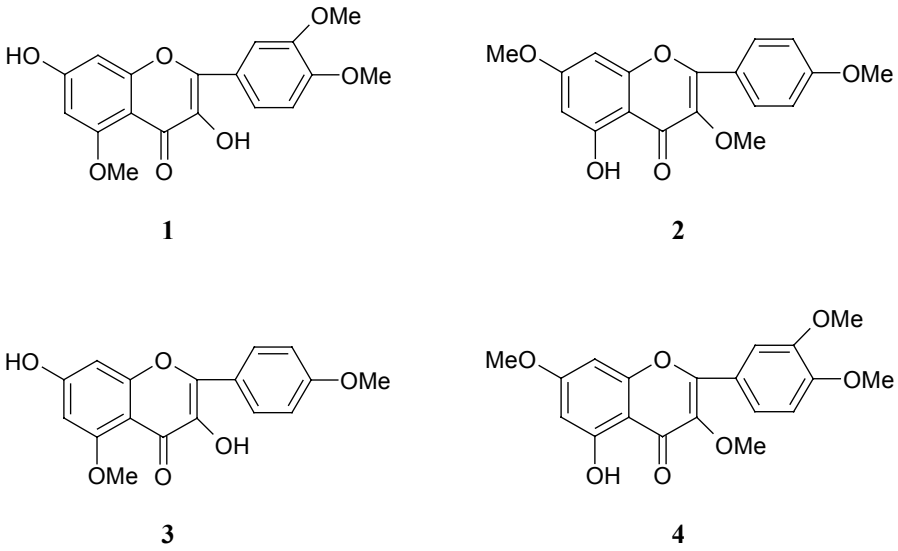


**Fig. 1**

Chemical structures of four methylated flavonols from *A. koenigii*

250-μm sieves. An accurately weighed portion (0.5 g) of each fine powder was extracted with 50 mL chloroform by heating under reflux for 4 h in a 100-mL round-bottomed flask. The chloroform extract was then concentrated to dryness and dissolved in 50 mL methanol. The methanol solution was filtered through a 0.45 μm membrane, then analyzed directly by HPLC.

## Chromatography

HPLC was performed with an Agilent 1100 system comprising a quaternary pump, online degasser, auto-sampler, column heater, and variable wavelength detector. Separation was achieved on a 4.6 mm × 150 mm, 5-μm particle, Zorbax XDB-$C_8$ reversed-phase analytical column (Agilent Technologies, USA). The mobile phase was acetonitrile containing 0.1% aqueous acetic acid; the amount of the latter component was changed linearly from 40 to 75% in 20 min. The flow rate was 1.0 mL $min^{-1}$. The elution profile was monitored and peaks were identified by UV absorbance at 360 nm. The temperature was maintained at 20°C. The injection volume was 10 μL.

## RESULTS AND DISCUSSION

### Optimization of the Method of Extraction

A variety of solvents, including chloroform, ethyl acetate, acetone, and methanol, were tested for extraction of the seeds of *A. koenigii* (sample no. 1). On the basis of the peak-area responses to these four compounds, chloroform was chosen as the best solvent. The method of extraction, including ultrasonic (4 × 15 min) and reflux (2 h or 4 h) extraction were then compared using chloroform as solvent. Reflux extraction for 4 h resulted in the best yield.

### Optimization of the HPLC Conditions

Different columns, mobile phases, and detection wavelengths were investigated during optimization of HPLC analysis. It was found that the Zorbax XDB-$C_8$ column enabled better peak separation than Zorbax SB-Phenyl, Alltima $C_8$, and Waters SunFire $RP_{18}$ columns. Addition of acetic acid to the mobile phase helped to generate the sharp peaks. The wavelength for detection of the compounds was selected by use of the diode-array detector. The UV spectra acquired for the compounds dissolved in the mobile phase are shown in Fig. 2. At 360 nm the separation was best, with a stable baseline and sharp peaks.

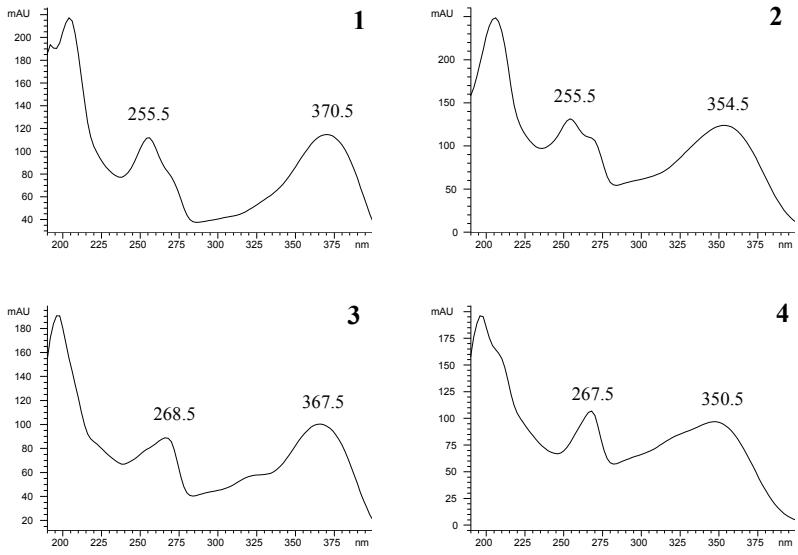


**Fig. 2**

UV spectra of the four methylated flavonols

## Calibration and Validation

Typical HPLC chromatograms obtained from reference standards and from plant samples are shown in Fig. 3. The retention times of **1**–**4** were 9.70, 10.90, 12.26, and 13.46 min, respectively.

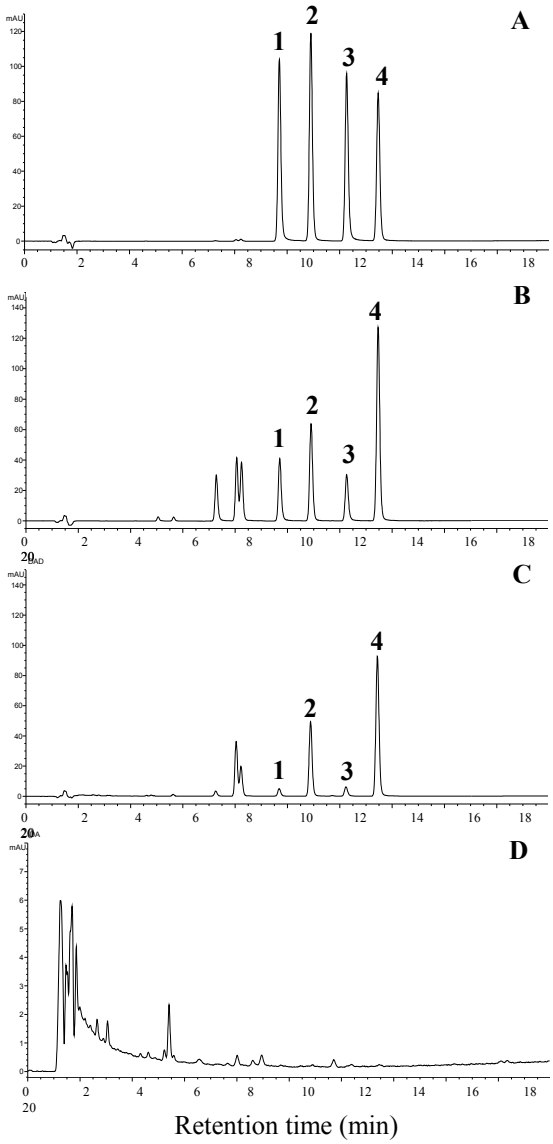


**Fig. 3**

Typical HPLC chromatograms: A, reference standards; B, seeds of *A. koenigii*; C, pericarp of *A. koenigii*; D, fruits of *A. tsao-ko*

A mixed stock solution consisting of standards **1** (0.387 mg $mL^{-1}$), **2** (0.281 mg $mL^{-1}$), **3** (0.224 mg $mL^{-1}$), and **4** (0.307 mg $mL^{-1}$) was prepared. Different volumes (0.02, 0.1, 0.2, 0.5, 1.0, and 2.0 mL) of this stock solution were then diluted to 10 mL with methanol to prepare calibration standards, the standards were chromatographed, and calibration plots were constructed. Good linearity was achieved in the ranges 0.774–77.4 μg $mL^{-1}$ for **1**, 0.562–56.2 μg $mL^{-1}$ for **2**, 0.448–44.8 μg $mL^{-1}$ for **3**, and 0.614–61.4 μg $mL^{-1}$ for **4**. The linear relationships between amounts injected (μg, *x*-axis) and peak-area ratio (*y*-axis) were expressed by the equations listed in Table I.

**Table I**

Calibration equations, *LOD*s, *LOQ*s, and reproducibility for the four analytes

| Compound | Calibration equation | Correlation coefficient | *LOD* (ng) | *LOQ* (ng) | Reproducibility (*RSD*, %) |
|---|---|---|---|---|---|
| **1** | $y = 2202.2x - 5.1832$ | 0.9998 | 0.46 | 1.24 | 1.96 |
| **2** | $y = 3490.7x - 1.1605$ | 0.9999 | 0.34 | 0.90 | 2.07 |
| **3** | $y = 3725.5x - 5.6725$ | 0.9998 | 0.27 | 0.72 | 1.81 |
| **4** | $y = 2394.0x - 0.6626$ | 0.9999 | 0.37 | 0.98 | 1.78 |

Serial dilutions of standard solutions of **1**–**4** were analyzed and the limits of detection (*LOD*) and quantification (*LOQ*) under the optimized chromatographic conditions were determined as the amounts for which the signal-to-noise ratios (*S*/*N*) were 3 and 10, respectively. The results indicated that the method enabled highly sensitive quantification of these four bioactive methylated flavonols in *A. koenigii* (Table I).

The seeds of *A. koenigii* (Sample no. 1) were extracted and analyzed in duplicate. The procedure was repeated five times to evaluate the reproducibility of the extraction procedure. The relative standard deviations indicated that reproducibility of extraction was good (Table I).

Standards of **1** (19.35, 38.70 μg $mL^{-1}$), **2** (14.05, 28.10 μg $mL^{-1}$), **3** (11.20, 22.40 μg $mL^{-1}$), and **4** (15.35, 30.70 μg $mL^{-1}$) were injected on the same day (intra-day; each concentration was injected five times within 24 h) and on different days (inter-day; each concentration was injected four times on each of five days with each injection separated by at least 24 h) to check the precision. The results, shown in Table II, indicate precision was good, with coefficients of variation for the intra-day and inter-day tests in the ranges 0.57–1.02% and 0.96–1.74%, respectively.

## Table II

Precision of analysis of the four analytes

| Compound | Concentration (μg $mL^{-1}$) | Mean (*RSD*) (%) | |
|---|---|---|---|
| | | Intra-day ($n$ = 5) | Inter-day ($n$ = 4) |
| **1** | 19.35 | 19.07 (0.74) | 18.99 (1.12) |
| | 38.70 | 38.29 (0.86) | 38.11 (0.98) |
| **2** | 14.05 | 14.13 (0.81) | 13.86 (1.03) |
| | 28.10 | 28.15 (0.92) | 28.22 (1.21) |
| **3** | 11.20 | 11.01 (0.57) | 10.95 (0.96) |
| | 22.40 | 22.59 (1.02) | 22.02 (1.58) |
| **4** | 15.35 | 15.23 (0.66) | 15.05 (1.74) |
| | 30.70 | 31.02 (0.94) | 30.43 (1.53) |

The accuracy of the method was evaluated by measurement of recovery. Four solutions were accurately spiked with known amounts of the reference compounds just before extraction of the seeds of *A. koenigii* (Sample no. 1). The results listed in Table III show recovery was good.

## Table III

Recovery of four analytes from *A. koenigii* ($n$ = 4)

| Compound | Contained (mg) | Added (mg) | Found (mg) | Recovery (%) | Mean recovery (%) | *RSD* (%) |
|---|---|---|---|---|---|---|
| **1** | 0.763 | 0.403 | 1.158 | 98.12 | 98.62 | 1.22 |
| | 0.765 | 0.403 | 1.159 | 97.65 | | |
| | 0.759 | 0.806 | 1.568 | 100.38 | | |
| | 0.772 | 0.806 | 1.565 | 98.33 | | |
| **2** | 0.732 | 0.416 | 1.155 | 101.57 | 99.36 | 1.62 |
| | 0.735 | 0.416 | 1.148 | 99.22 | | |
| | 0.729 | 0.832 | 1.542 | 97.72 | | |
| | 0.742 | 0.832 | 1.565 | 98.92 | | |
| **3** | 0.356 | 0.194 | 0.543 | 96.29 | 98.01 | 1.43 |
| | 0.358 | 0.194 | 0.547 | 97.65 | | |
| | 0.355 | 0.388 | 0.741 | 99.61 | | |
| | 0.361 | 0.388 | 0.743 | 98.50 | | |
| **4** | 2.308 | 1.051 | 3.351 | 99.26 | 99.37 | 2.07 |
| | 2.317 | 1.051 | 3.391 | 102.23 | | |
| | 2.297 | 2.102 | 4.369 | 98.58 | | |
| | 2.338 | 2.102 | 4.385 | 97.40 | | |

### Determination of the Four Methylated Flavonols in Samples

Seeds and pericarp of *A. koenigii* and fruit of *A. tsao-ko* were extracted as described above and analyzed by HPLC. The results obtained are summarized in Table IV.

### Table IV

Amounts of the four compounds in *A. koenigii* and related sample

| Sample no. | Species | Part | Location | Content (%, $n = 3$) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | **1** | **2** | **3** | **4** | Total |
| 1 | *A. koenigii* | Seed | Napo, Guangxi | 0.152 | 0.146 | 0.071 | 0.460 | 0.829 |
| 2 | *A. koenigii* | Seed | Yuanyang, Yunnan | 0.138 | 0.137 | 0.067 | 0.434 | 0.776 |
| 3 | *A. koenigii* | Pericarp | Napo, Guangxi | 0.010 | 0.058 | 0.008 | 0.167 | 0.243 |
| 4 | *A. koenigii* | Pericarp | Yuanyang, Yunnan | 0.013 | 0.052 | 0.011 | 0.164 | 0.240 |
| 5 | *A. tsao-ko* | Fruit | Jinghong, Yunnan | –[a] | – | – | – | – |

[a]Not detected

The results showed there was little difference between the amounts of these constituents in samples from two different regions. The total amounts of the compounds in the seeds (0.776–0.829%) were obviously higher than in the pericarp (0.240–0.243%). Compounds **1** and **3** were present in small quantities, approximately 0.01%, in the pericarp and much lower than that in the seeds. These data also confirmed that retusin (**4**) was the major constituent – almost 0.50% in the seeds of *A. koenigii*. Considering the bioactivity of retusin, use of this plant resource is very promising.

None of these four methylated flavonols were detected in the fruit of *A. tsao-ko*, however. When the HPLC chromatograms obtained from *A. tsao-ko* (Fig. 3D) and *A. koenigii* (Fig. 3B) are compared, substantial differences are apparent. From these distinct chemical profiles it does not seem reasonable to use *A. koenigii* as a substitute for Fructus Tsaoko.

## CONCLUSION

For the first time a simple and rapid HPLC method has been deve-

loped for simultaneous analysis of four major bioactive methylated flavonols in *A. koenigii*. The assay is accurate, reproducible, and sensitive. The method has been successfully used for analysis of these four compounds in different samples. It was found that the samples furnish similar, characteristic HPLC chromatograms, thus making the method useful for quality control of the crude drugs. The results also indicate this medicinal plant could possibly be used as a source of these compounds.

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