· Research Papers ·

Quantitative Metabolomics: Analysis on Active Components in Extracts from *Kaki Folium*

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Abstract: **Objective** In order to analyze the active components in the extracts from *Kaki Folium* (KF), quantitative metabolomics approach was adopted to investigate the number of active components existing among the different extracts and their variation. **Methods** LC-MS method was established for the quantitative determination of the active components taking the mixture with reference substance as tested sample. **Results** In terms of the number of active components and amount presented in the different tested samples of KF extracted by many types of solvents, variation was observed. But rutin, astragalin, and kaempferol were presented in all samples. Difference was found between the samples extracted from the products on market and from the raw materials of KF processed by polar solvents with different recipes. However, the three active components were found in all samples examined. **Conclusion** These results might be valuable as all information and could be used for the optimization of raw materials extraction procedure to enhance the productivity.

Key words: active components; astragalin; extracts from *Kaki Folium*; kaempferol; metabolomics; quantitative metabolomics; rutin
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Introduction

Persimmon (*Diospyros kaki* L. f.) distributes widely in China, Japan, Korea, and Thailand. In Europe, it was introduced in the 17th century and later. In the 18th century, it was already known worldwide. *Kaki Folium* (KF, the leaves of *D. kaki*) contains proteins, sugars, flavonoids, amino acids, and vitamins, as well. The components in KF have wide range of pharmacological activities, including antibacterial, anti-inflammatory, blood pressure-lowering, lipid-lowering, anti-allergenic, antiviral, and anticancer activities. It could also promote the body metabolism, relieve cough, and dispel phlegm. Flavonoids from KF have attracted public attention (Hertog *et al*, 1993; Maxwell, Cruickshan, and Thorpc, 1994) because flavonoids in tea, fruits, and vegetables could lessen the risk of cardiovascular diseases, which could be used to prepare drugs for the prevention and treatment of atherosclerosis, hypertension, and coronary heart disease (Sugiura, 1997; Sorilano *et al*, 2006). A novel and patented Chinese medicine, made of the extract from *Kaki Folium* (EKF), mainly contained flavonoids, including quercetin, kaempferol and their glucosides (astragalin, rutin, and hyperin) (Bei *et al*, 2005), and some phenolic acids (protocatechuic acid, benzoic acid, salicylic acid, and syringic acid) (Zhang *et al*, 1983). This herbal medicine has been used for the treatment of stroke or syndrome of apoplexy in clinic to improve the outcome of ischemic stroke for years in China. Moreover, the remedy has been reported to possess the

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significant efficacy and few side effects in the treatment of stroke patients, such as cerebral atherosclerosis, transitory ischemic syndrome, cerebral thrombogenesis, cerebral thrombosis sequelae, apoplexy sequelae, cerebral embolism. The clinical application and marketing for nearly 20 years proved that the tablets had well-accepted effectiveness in the treatment of cerebral-cardiovascular diseases. In the practice, new natural drug development from single medicinal plant is consistent with international practice (Zhang *et al*, 1983).

The fruit part of some plants in *Diospyros* L. contains chemicals used as fish poisoning agents or medicines, although the active components have not yet been identified. The edible fruit contained high content of tannin, but there were only a few fruits used as fish poisoning agents and medicines (Utsunomiya *et al*, 1998). *D. kaki* is widely distributed in both North and South China. While KF contains starch, sugar, protein, flavonoids, amino acids, and vitamins. Flavonoids are phenolic substances including over 8000 individual known components isolated from a wide range of plants. Many studies have suggested that the flavonoids could exhibit the biological activities, such as anti-allergenic, antiviral, anti-inflammatory, and vasodilating activities (Pietta, 2000). Previous studies have demonstrated that the flavonoid glucosides extracted from KF could lower blood pressure, increase coronary blood flow in anaesthesized dog and isolated rabbit heart, and dilate the rabbit ear vessels (PLA 58th Hospital, 1973). It was reported that flavonoids isolated from KF had a hypotensive activity on rats and inhibitory effects on anginotensin-converting enzyme (Kameda *et al*, 1987). Flavonoids from KF (FKF) are main constituents in Naoxinqing Tablets (NXQ), and account more than 25% of the total components in NXQ extracts. Although there were some reports on the pharmacological effects of NXQ and the other flavonoids mentioned above, little was known about how FKF worked in neuronal cells. Here we carried out the present study to examine the effects of FKF on H_2O_2-induced NG108-15 cell injury (Bei *et al*, 2005).

The EKF and its active components have extensive biological and pharmacological effects. In a review, the relative literatures about biological and pharmacological investigation on EKF and its active components were introduced and analyzed (Yin *et al*, 2010b), so were the relative literatures about pharmacokinetic investigation in another review introduced and analyzed (Yin *et al*, 2010a).

Based on the concept of quantitative metabolomics, quantitative determination of the active components in EKF was carried out by LC-MS technology. This attempt may be valuable and be used as the guidance for the optimization of bioactive components extraction.

Materials and methods

Test samples

Among eight samples examined, six were extracts from *Kaki Folium* (No. 1－6), the other two were intermediates of Yuannao Xinqing Pills extracted by two processing approaches (No. 7 and 8), and all samples were kindly provided by Hutchison Whampoa Guangzhou Baiyunshan Chinese Medicine Co., Ltd. (China).

Reference substances

There are quercetin, kaempferol, rutin, astragalin, isoquercetin, myricetrin, hyperin, protocatechuic acid, succinic acid, furonic acid, malic acid, salicylic acid, benzoic acid, syringic acid, oleanolic acid, ursolic acid, protocaechuic aldehyde, and β-sitosterol as reference substances, which were purchased from National Institute for Food and Drug Control (Beijing, China).

Equipments

LCQ LC-MS (Thermo Finnigan Co., US) was used, equipped with UV-DAD detector and ESI source. Surveyor Pump and Autosampler were applied. Xcalibur 1.4 Workstation was used for data processing. Vortex was purchased from Shanghai Global Scientific Equipment Co., Ltd. Pipette was from Dalong Healthcare Apparatus (Shanghai) Co., Ltd. (China).

Test reagents

Methanol and acetonitrile of HPLC grade were purchased from Tianjin Concord Tech. Reagent Co., Ltd. Deionized water was self-made.

LC-MS^n analysis

Chromatography was carried out on Diamosil C_{18} column (250 mm × 4.6 mm, 5 μm); And column temperature was 30 ℃. The elution solvent was composed of methanol (A) and aqueous ammonium formate buffer at 20 mmol/L (B). The gradient program was as follows: 0—10 min, 5% A; 10—50 min, 10% A; 50—70 min, 95% A; 70—75 min, 5% A. The flow rate was 0.4 mL/min.

Preparation of reference substance solution

Eighteen reference substances (approximately 1 mg each) were weighed and dissolved in deionized water, methanol, or mixed solution with equal volume. Each reference (20 μL) solution with the identical tag was mixed and loaded. Each sample was scanned both in positive and negative ion modes, and then detected by UV-DAD. The corresponding concentration and solvents were presented in Table 1.

Table 1 Concentration and solvents of reference substances

Reference substances	Concentration / ($mg \cdot mL^{-1}$)	Solvents
syringic acid	1.15	H_2O-MeOH (50:50)
protocaechuic aldehyde	1.06	H_2O-MeOH (50:50)
succinic acid	1.14	H_2O
furonic acid	1.12	H_2O
malic acid	1.13	H_2O
myricetrin	1.17	MeOH
benzoic acid	1.01	MeOH
quercetin	1.05	MeOH
oleanolic acid	1.14	MeOH
salicylic acid	1.30	MeOH
isoquercetin	1.18	MeOH
ursolic acid	1.20	MeOH
protocatechuic acid	1.11	H_2O-MeOH (50:50)
β-sitosterol	1.02	MeOH
rutin	1.05	MeOH
hyperin	1.15	MeOH
astragalin	1.03	MeOH
kaempferol	1.02	MeOH

Results

Spectra of reference substances and data analysis

By comparison with UV absorption and full-scanned mass spectra in positive and negative ion modes, retention time (t_R) and corresponding respond values of 13 reference substances were obtained (Table 2). LC-UV-MS analyses of 13 reference substances were carried out. These analyses included full scanning of positive and negative ions, UV spectrum, and MS at peak position. LC-UV-MS analyses of the two reference substances, quercetin and protocatechuic acid, were shown in Figs. 1 and 2, respectively. LC-UV-MS analyses of three batches of mixed reference substances were presented in Fig. 3.

Sample analysis of EKF

Each of right analytes (1 g) was vortexed and dissolved in 10 mL of solvent to a final concentration of 100 mg/mL. Each sample (10 μL) was loaded, scanned both in positive and negative ion modes, and then detected by UV-DAD. By comparison with LC-UV-MS analysis of blank solvent and response values on MS, 11 known components were found in the samples, and their corresponding concentration was preliminarily determined. The contents of 11 determined components of eight samples in the extracts were shown in Table 3. According to chromatographic and MS information obtained from LC-MS analysis, the peak areas of each possible component in three samples were over 100.

Table 2 Chromatographic and MS data of reference substances

No.	Reference substances	Concentration / ($mg \cdot mL^{-1}$)	t_R / min	*m/z*		Peak areas
				+	−	
1	β-sitosterol	1.02	56.99	414.91		254 161 910
2	rutin	1.05	42.58	610.79		714 002 206
3	astragalin	1.03	45.11	448.77		666 546 970
4	isoquercetin	1.18	42.82	464.79		654 000 956
5	hyperin	1.15	42.83	464.81		663 716 250
6	protocaechuic aldehyde	1.06	33.36		137.51	68 682 377
7	succinic acid	1.14	6.20		117.34	1 315 783
8	malic acid	1.13	6.09		133.18	2 218 835
9	quercetin	1.05	48.56		301.31	37 556 325
10	salicylic acid	1.30	32.59		137.36	61 857 816
11	protocatechuic acid	1.11	8.26		153.30	10 812 782
12	kaempferol	1.02	51.71		285.45	131 368 052
13	myricetrin	1.17	45.10		137.31	9 676 162

Isoquercetin and hyperin could not be distinguished, due to t_R and ion mass of both reference substances were very close

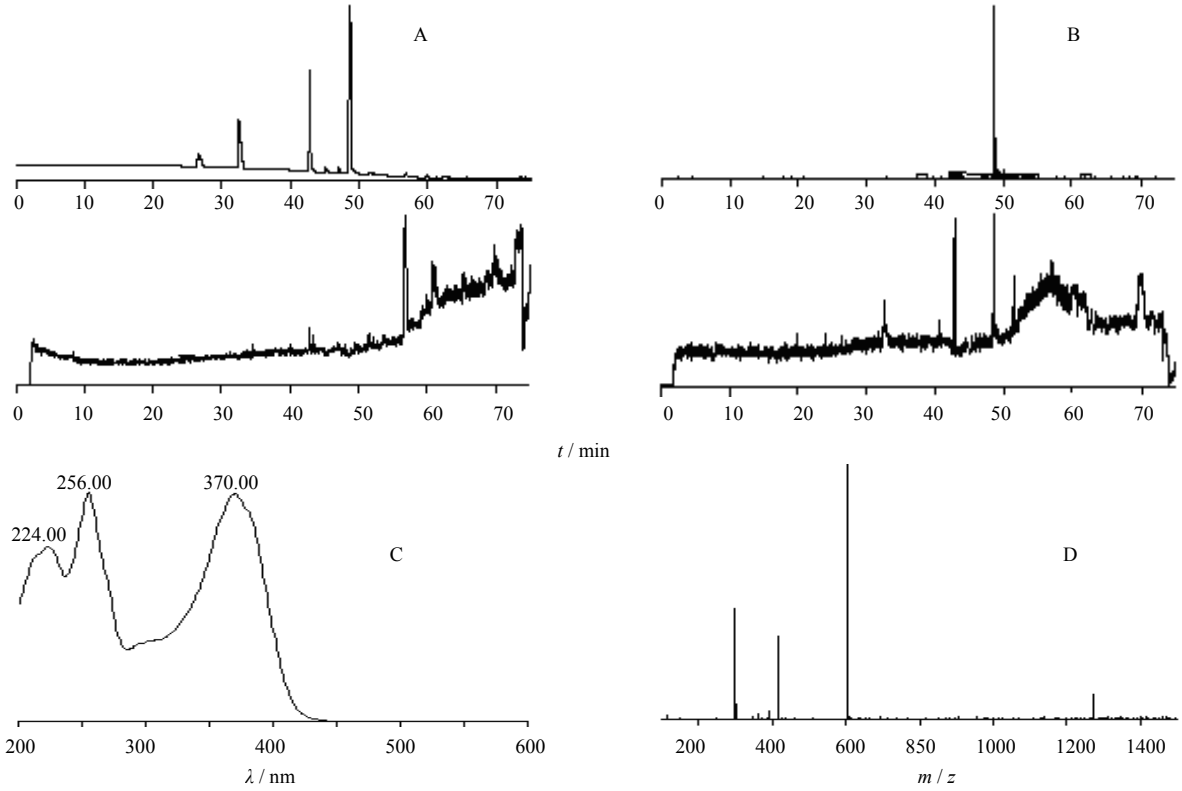

Fig. 1 LC-UV-MS analysis of quercetin

A: full scanning of positive ion B: full scanning of negative ion C: UV spectrum at peak position D: MS at peak position

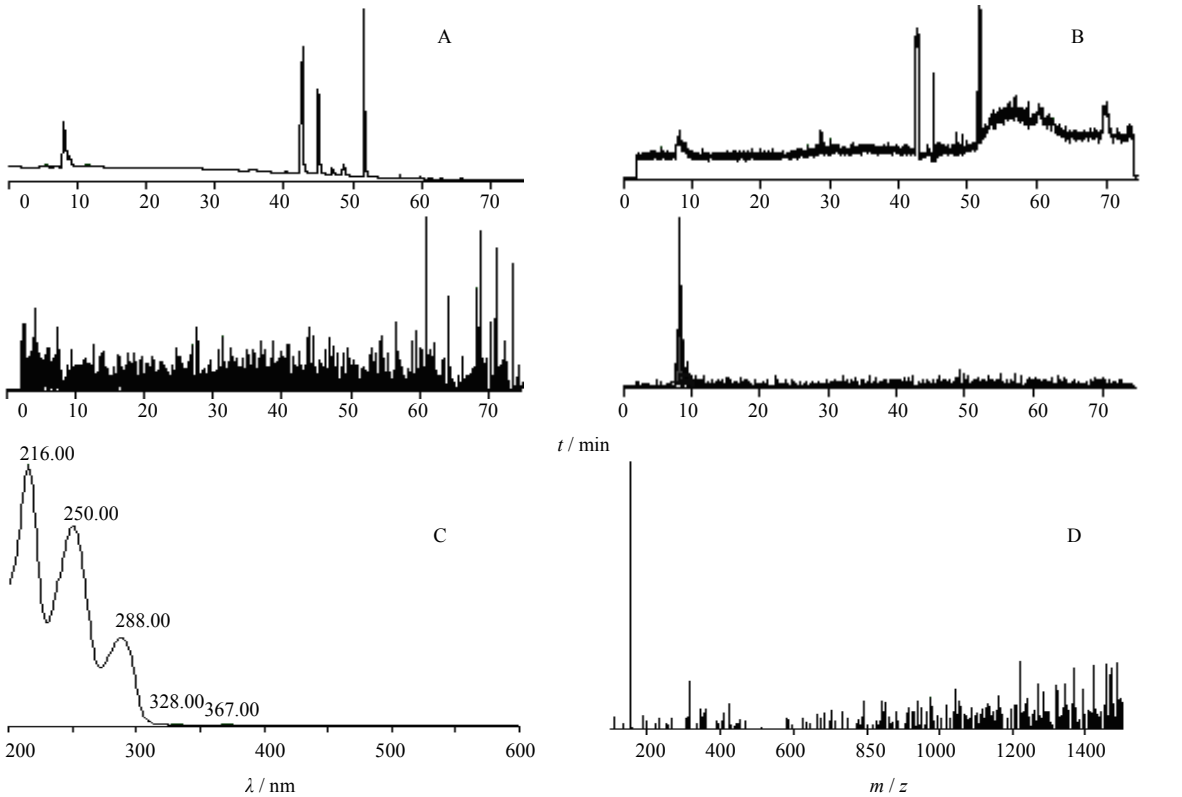

Fig. 2 LC-UV-MS analysis of protocatechuic acid

A: full scanning of positive ion B: full scanning of negative ion C: UV spectrum at peak position D: MS at peak position

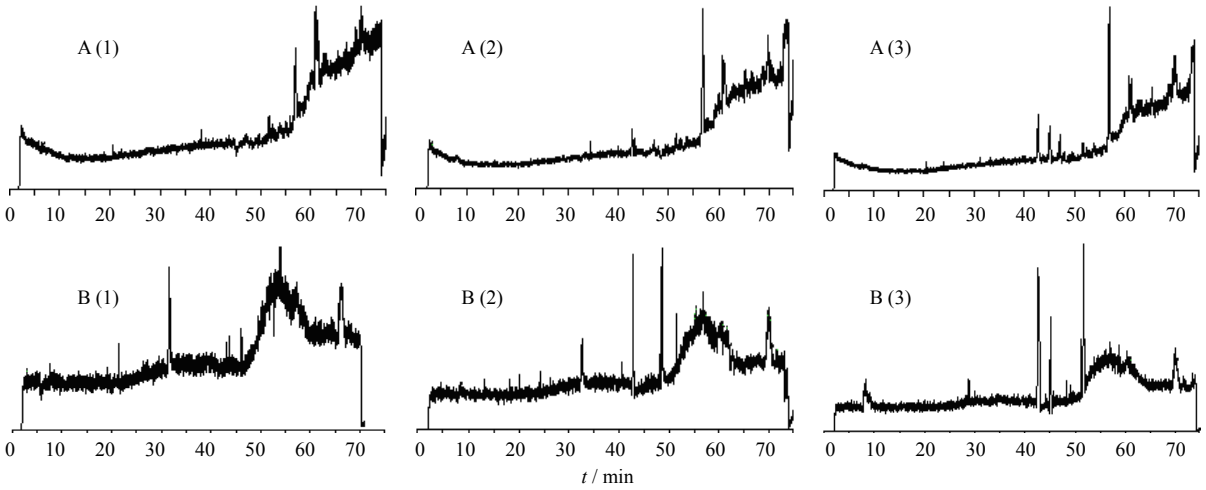

Fig. 3 LC-UV-MS analysis of mixed reference substances

A: full scanning of positive ion B: full scanning of negative ion 1－3: mixed reference substances 1－3

Table 3 Determination of 11 components in eight tested samples

Samples	Content / ($\mu g \cdot g^{-1}$)										
	β-sitosterol	rutin	astragalin	protocaechuic aldehyde	succinic acid	malic acid	quercetin	salicylic acid	protocatechuic acid	kaempferol	myricetrin
1	30.8	74.4	490	0.105	6.99	45.9	0	0.259	0	0.878	2.11
2	111	58.9	1057	0	0	11.6	0	0	0	6.64	5.04
3	75.1	80.7	1141	0.085	0	0	21.3	0.138	0.833	15.3	11.4
4	0	56.3	23.7	0.211	0	0	0.348	0.239	0	0.223	0
5	209	36.1	826	0	0	10.2	0	0	0	9.87	13. 9
6	127	57.7	973	0.088	0	0	8.77	0	0	8.49	3.58
7	185	65.8	2878	6.36	0	0	145	8.66	0	58.0	108
8	0	34.8	2979	4.76	0	0	158	7.32	17.2	76.8	141

The column distribution was plotted in terms of molecular weight (MW) of these possible components. As shown in Fig. 4, compound numbers were 10, 46, 21, 29, and 21 with MW of 100－200, 200－300, 300－400, 400－500, and 500－600, respectively.

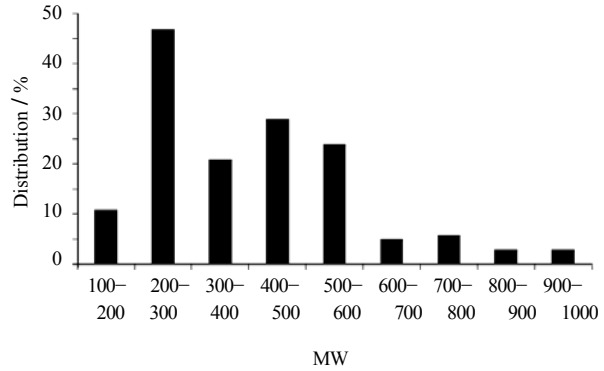

Fig. 4 MW distribution of components in EKF

Discussion

The recent development of the metabolomic technologies and quantitative metabolomic methods typically based on GC-MS, LC-MS, and NMR is suitable for the large-scale measurement of metabolite levels in human, animal, and plant (Urakami *et al*, 2010; Hirayama *et al*, 2009; Kastenmuller *et al*, 2011; Lanza *et al*, 2010; Ando *et al*, 2010). Various types of chemicals could be produced in the course of the growth, development, apoptosis, and related metabolism process of plant cells, namely secondary metabolites, which is considered as the ground for research on their bioactivities.

It could be seen that the number and amount of active components presented variously, with respect to the samples of KF extracted by different polar solvents.

In addition, the differences between the samples

extracted from the products on market and from the raw materials of KF were observed. Moreover, three bioactive components, rutin, astragalin, and kaempferol, were presented in all the samples examined, regardless of the solvents chosen for processing. The results might be very helpful for the optimization of raw material extraction to enhance the productivity of bioactive components.

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