



# Determination of flavonol glycosides in green tea, oolong tea and black tea by UHPLC compared to HPLC



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## ABSTRACT

An UHPLC method for the determination of flavonol glycosides (FOG) from green and oolong tea vs. black tea has been developed for the first time. Sample clean-up method by means of polyamide column chromatography was optimized with multiple-step elution. Using UHPLC and HPLC with gradient elution and photodiode array detection, eighteen FOG compounds were determined with the aid of electrospray tandem mass spectrometry. These FOG compounds were qualified on both UHPLC and HPLC, and this UHPLC method successfully separated rutin (quercetin-3-O-rutinoside) and K-grg (kaempferol-3-O-glucorhamnogluconide) while conventional HPLC method did not. The total amounts of FOG compounds in the tea samples were 2.32–5.67 g/kg dry weight (calculated as aglycones), and there is no significant difference for the total FOG content among green tea, oolong tea and black tea. However, kaempferol glycosides are more abundant in green teas, while oolong tea has more quercetin and myricetin glycosides. In black tea quercetin glycosides were most abundant.

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## 1. Introduction

Tea (*Camellia sinensis* L.O. Kuntze) is the most popular flavored and healthy beverage beyond water in the world. The flavor and taste of tea brews depends on the chemical constitutions, although it is still difficult to illustrate. There are many kinds of compounds indispensable for tea flavor or taste, including catechins, theaflavins, flavonols, some other flavonoids, theanine, proteins and constructive amino acids, sugars and polysaccharides, lipids, caffeine and other methylxanthines, minerals, etc. (Harbowy, Balentine,

Davies, & Cai, 1997). Flavonol glycosides (FOG) are one of the most important groups of polyphenols besides catechins in tea. They are of interest because of their physiological activity (Matsubara et al., 1985; Suzuki, Honda, Funatsuki, & Nakatsuka, 2002), the important effect on tea brew color and taste (Hofmann, Scharbert, & Stark, 2006).

FOG were firstly detected in tea in the 1950s, after extraction and separation by two-dimensional thin layer chromatography (TLC) (Nakabayashi, 1953; Oshima & Nakabayashi, 1953; Roberts, Cartwright, & Wood, 1956). Determination of FOG was carried out firstly by spectrophotometry, followed by HPLC, assisted with the structural elucidation of flavonol rhamnosyldiglycosides by NMR. FOG compounds derived from quercetin and kaempferol aglycones were isolated and identified (Finger, Engelhardt, & Wray, 1991a, 1991b). Some new FOG compounds from tea derived from myricetin aglycone were also detected (Hilal & Engelhardt, 2009). Unlike catechins where an ISO standard exists (ISO, 2005; Stodt & Engelhardt, 2013) there is no standard method for the determination of FOG in tea published. Consequently, not too many data can be found in the literature. A HPLC method was established to determine 16 kinds of FOG, including flavonol mono-, di- and triglycosides, from white teas and green teas based on the polyamide clean-up treatment (Engelhardt, Finger, Herzig, &

**Abbreviations:** FOG, flavonol-3-O-glycosides; glu, glucoside; gal, galactoside; M-rdg, myricetin rhamnodigluconide; M-rut, myricetin rutinoside; M-gal, myricetin galactoside; M-glu, myricetin glucoside; Q-grg, quercetin glucorhamnogluconide; Q-rdglu, quercetin rhamnodigluconide; Q-rrg, quercetin dirhamnogluconide; Q-rgal, quercetin rhamnogalactoside; Q-rut, quercetin rutinoside; Q-gal, quercetin galactoside; Q-glu, quercetin glucoside; K-drgal, kaempferol dirhamnogalactoside; K-grg, kaempferol glucorhamnogluconide; K-rdg, kaempferol rhamnodigluconide; K-gal, kaempferol galactoside; K-drglu, kaempferol dirhamnogluconide; K-rut, kaempferol rutinoside; K-glu, kaempferol glucoside; WLLJ, West Lake Longjing green tea; LSYW, Lushan Yunwu green tea; SBXT, Sanbeixiang green tea; DAOT, Dense aroma oolong tea; FSOT, faint scent oolong tea; YNBT, Yunnan black tea; ASBT, Assam black tea; KEBT, Kenya black tea.

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Kuhr, 1992; Hilal, 2010). The content of FOG from green tea and white tea samples in the assay could reach up to 5453 and 4071 mg/kg.

Some recent investigations were published based on high performance liquid chromatography combined with ultraviolet (UV), mass spectrometric detection (MS), LC-LTQ-Orbitrap Fourier transformed (FT)-MS and LC time-of-flight-(TOF)-MS coupled to solid-phase extraction (SPE) NMR. The composition and content of up to 177 tea phenolic compounds from selected green, green pu-erh, black, white and oolong teas, including catechins, flavonol and flavones glycosides, phenolic acids, purine alkaloids, and glycosylated and acetylated derivatives of flavan-3-ols and flavonols were annotated (van der Hooft et al., 2012; Wu, Xu, Héritier, & Andlauer, 2012). However, Ultra High Performance Liquid Chromatography (UHPLC) has not been used for the quantitative determination of the flavonol glycosides in tea.

The aim of this work was to establish a method for the quantitative determination of FOG from different types of tea by UHPLC vs. HPLC. The method should include a reliable clean-up for tea samples. This is especially necessary for green teas where otherwise mass spectrometric measurements might be interfered by high concentrations of co-eluting catechins.

## 2. Material and methods

### 2.1. Standard compounds and other chemicals

Rutin trihydrate approx. 95% was purchased from Sigma Chemical Company (St. Louis, USA), while myricitrin, orientin, apigenin-7-glucoside, kaempferol-3-rhamnosidoglucoside, and luteolin-3',7-diglucoside were obtained from Roth (Karlsruhe, Germany).

### 2.2. Tea samples

Commercial samples of green tea, oolong teas and black teas were used. 3 green teas (Westlake Longjing tea, Sanbeixiang green tea, Lushan Yunwu tea), 2 oolong teas (Dense aroma Tieguanyin, Faint scent Tieguanyin) and 1 Yunnan black tea from China were collected by Tea Research Institute, Chinese Academy of Agricultural Sciences. 2 black teas from Kenya and India were collected by Institute of Food Chemistry, Technische Universität Braunschweig. Ground tea (2.5 g, particle size < 0.5 mm) was extracted with 200 mL of methanol at 40 °C for 15 min using a rotary evaporator without vacuum, then filtered with a Buchner funnel. The extraction was repeated twice with each 75 mL of 70% methanol aqueous solution, the filtered solutions were combined, and the pooled filtrates were concentrated to about 40 mL to remove methanol completely, and made up to 100 mL with water.

### 2.3. Polyamide column chromatography

For pretreatment of polyamide SC 6, particle size 0.05–0.16 mm, (Macherey & Nagel, Dueren, Germany) see Engelhardt et al. (1992). A glass column (14 cm × 2.5 cm i.d.) was filled with 10 cm-layer polyamide and conditioned with 250 mL of deionized water. Tea extract (25 mL) was applied to the column. After washing with 100 mL of water, the phenolic compounds were eluted with 250 mL of methanol. The methanol was removed using a rotary evaporator, and the residue was transferred with 4 mL of N, N-dimethylformamide (DMF) to a 10 mL measuring flask and made up to volume with water. DMF is known to be a good solvent for FOG. After membrane filtration (0.2 µm) the solution was ready for UHPLC.

The new method of multiple-step elutions was presented after modification experiments with 20%, 50%, 60%, 70%, and 80% methanol elution. In the new clean-up method, one more elution with 200 mL of 60% methanol was inserted right after elution with 100 mL of water, and other steps were not changed.

### 2.4. HPLC–DAD analysis of flavonol glycosides

For HPLC analysis the following equipment was employed: Agilent Technologies series 1100 HPLC consisting of autosampler, binary pump, column oven, and DAD detector. The measurement was performed using a modified system by Hilal (2010): flow rate 1.0 mL/min; temperature 20 ± 0.5 °C; column: Phenomenex Aqua 5 µ, C18 125A 250 × 4.60 mm (Phenomenex, Aschaffenburg, Germany); injection volume, 20 µL; mobile phase, A = 2% acetic acid (aq), B = acetonitrile; gradient elution, 6–17% B, 0–28 min; 17–20% B, 28–53 min; 20% B, 53–65 min; 20%–100% B, 65–66 min; 100% B, 66–76 min; 100%–6% B, 76–77 min; equilibration time, 20 min; detection, diode array (λ = 278 and 354 nm). All eluents used were HPLC grade (VWR International S.A.S.).

### 2.5. UHPLC–DAD analysis of flavonol glycosides

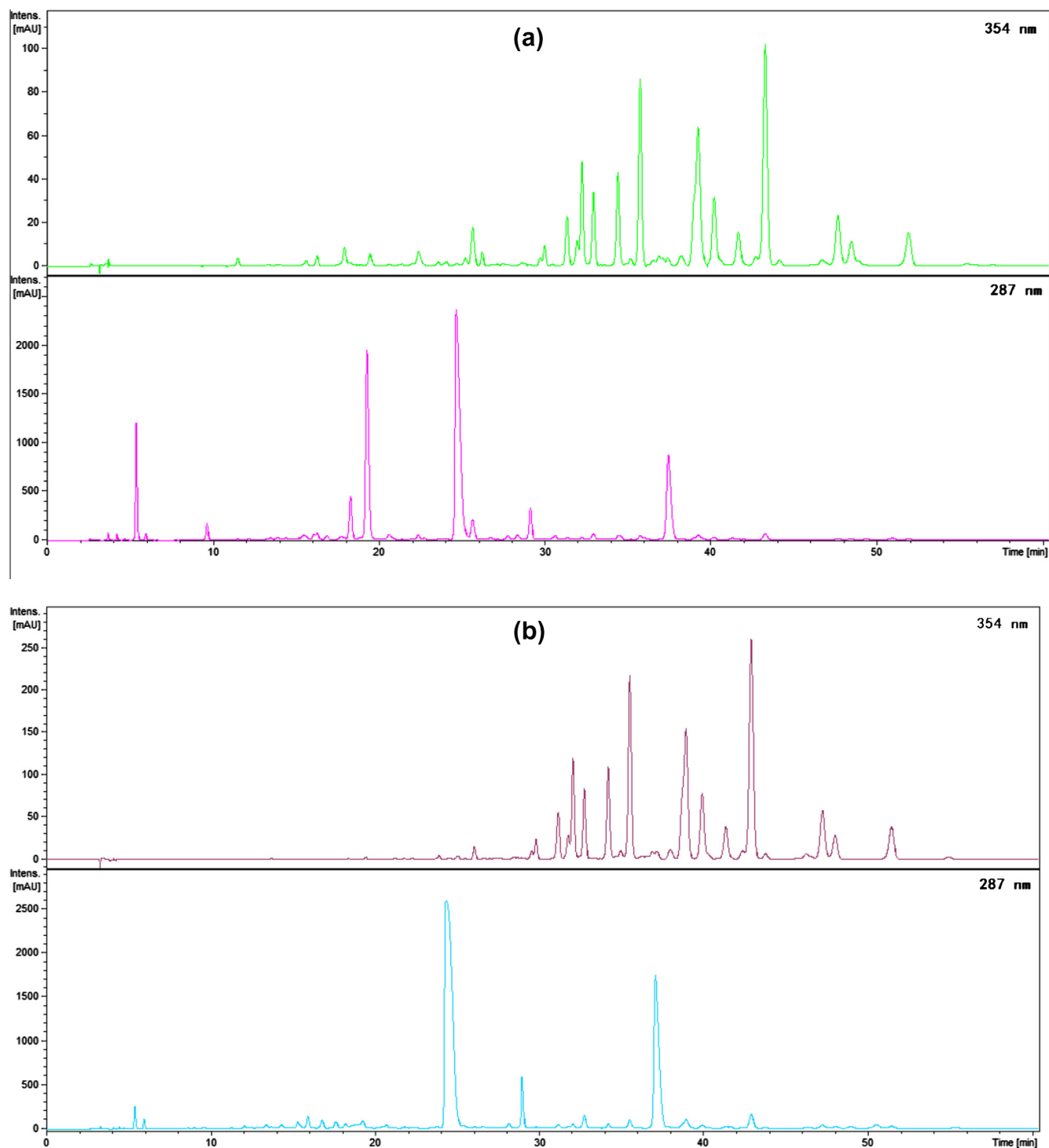
For UHPLC analysis the following equipment was employed: Agilent Technologies series 1290 Infinity UHPLC consisting of autosampler, binary pump, column oven, and DAD detector. The measurement was adjusted as follows: flow rate, 0.4 mL/min; temperature, 20 ± 0.5 °C; column, Agilent ZORBAX Eclipse PLUS C18, Rapid Resolution HD 2.1 × 100 mm 1.8 µ; injection volume, 5 µL; mobile phase, A = 2% acetic acid (aq), B = acetonitrile; gradient elution, 6–17% B, 0–5 min; 17–20% B, 5–9 min; 20% B, 9–11 min; 20%–100% B, 11–12 min; 100% B, 12–15 min; 100%–6% B, 15–16 min; equilibration time, 4 min; detection, diode array (λ = 278 nm to detect catechins and 354 nm for FOG determination). All eluents used were HPLC grade (VWR International S.A.S.).

### 2.6. Analysis via HPLC–ESI–MS<sup>n</sup>

The separation via HPLC was performed on an Agilent Technologies series 1100 HPLC consisting of binary pump and DAD detector with a series 1200 autosampler: flow rate, 0.4 mL/min; temperature, RT; column, Phenomenex Aqua 5 µ, C18 125A, 250 × 2.00 mm (Phenomenex); injection volume, 5 µL; mobile phase, A = 2% acetic acid (aq), B = acetonitrile; gradient elution, 6–17% B, 0–28 min; 17–20% B, 28–53 min; 20% B, 53–65 min; 20%–100% B, 65–66 min; 100% B, 66–76 min; 100%–6% B, 76–77 min; equilibration time, 20 min; detection, diode array (λ = 278 for catechins and 354 nm for flavonol glycosides). All eluents used were HPLC grade (Baker, Gross-Gerau, Germany). The analysis via ESI–MS<sup>n</sup> was done on a Bruker Daltonics (Bremen, Germany) HCT PTM Discovery System electrospray ionization ion trap mass spectrometer using the following parameters: negative ion mode; scan, 100–1000 *m/z*; nebulizer, 60.00 psi; dry gas, 11 L/min; 365 °C; capillary, ±3500 V. The mother ions and fragmentation ions were used to the identification of flavonol glycosides.

### 2.7. Quantification

For clean-up and UHPLC see above. Rutin trihydrate (0.52–103.7 mg/L, calculated as quercetin), myricitrin (0.99–197.4 mg/L, calculated as myricetin) and kaempferol-3-rhamnosidoglucoside (0.96–96.3 mg/L, calculated as kaempferol) were used for calibration purposes. Tea samples were analyzed with and without addition of a standard solution containing known amounts of rutin, myricetin and kaempferol.



**Fig. 1.** HPLC chromatogram of green tea sample: (a) before polyamide column chromatography clean-up (upper trace, 354 nm; lower trace, 287 nm); (b) after polyamide column chromatography clean-up (upper trace, 354 nm; lower trace, 287 nm).

## 2.8. Detection limit

Using solutions of rutin, myricetin and kaempferol-3-rhamnosidoglucoside (7 points in 0.05–10.97 mg/L, calculated as aglycones), the minimal levels at which reliable UV-spectra could be obtained using the DAD were determined and 0.2 mg/L was defined as the detection limits.

## 3. Results and discussion

### 3.1. Flavonol glycosides and catechins in green tea and oolong tea

There are many catechins in tea, especially in green tea and oolong tea. Green tea usually contains 20–30% of catechins (Engelhardt, 2010; Wang, Provan, & Helliwell, 2000), oolong tea

**Table 1**

The separation of catechins and FOG with 3 steps of elution on polyamide column chromatography.

Elution	Peak area at 287 nm (mainly for catechins class)		Peak area at 354 nm (mainly for FOG compounds)	
	mAu * s	%	mAu * s	%
Water	61,773	22.42	64	0.25
60% methanol	62,198	22.57	24,076	95.87
100% methanol	151,563	55.01	974	3.88

has the similar amount, while black tea only contains less than 10% due to the transformation into theaflavins and thearubigins. According to the ISO database compared to catechins, FOG are much less abundant in green, white and oolong tea with an average amount of up to 5453 and 4071 mg/kg (Hilal, 2010). Other data in the literature are a bit different, e.g. Engelhardt et al. (1992) detected 1837–9621 mg/kg (average:  $n = 29$ , data given as aglycones). So, the content of catechins is up to 50 times than the FOG content in green tea, while in some black tea samples the sum of FOG is higher compared to the sum of catechins. In another study with more than 50 green and black teas each the contents were as follows: green teas 0.28–0.95%, average 0.64, black teas 0.24–0.87%, average 0.47%, both calculated as aglycones (Engelhardt, Lakenbrink, & Lapczynski, 2000).

Fig. 1 shows the result of polyamide column chromatography clean-up treatment. All FOG compounds have a strong absorbance around 350 nm, in contrast to catechins. Fig. 1 demonstrates that all the FOG compounds are recovered by polyamide column chromatography comparing the two chromatograms at 354 nm.

Catechins, caffeine, gallic acids and some other related compounds have a strong absorbance around 280 nm, while FOG have less effects due to the lower absorbance at this wavelength and the at least in green tea smaller content. It could be concluded that polyamide clean-up could keep all the main catechins and reduce most gallic acids and caffeine.

Polyamide clean-up had been successfully used in the determination of black tea samples (Engelhardt et al., 1992; Hilal, 2010). But for green tea or oolong tea, the polyamide clean-up method might be modified in order to provide more powerful support for the structural identification and qualification.

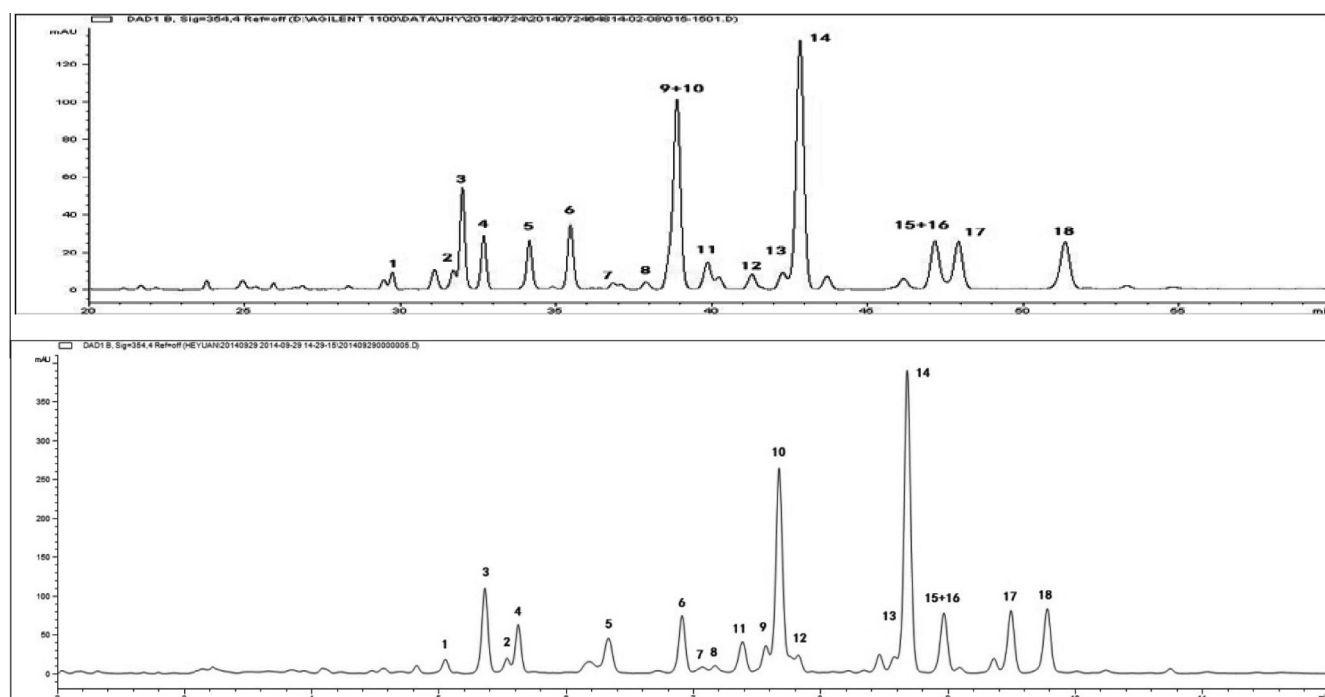
### 3.2. Clean-up with polyamide column chromatography

Polyamide column chromatography is a good way for clean-up of tea samples, and was used to analyze tea samples. The common method is a two-step of elution, firstly a washing step with pure water, then with 100% methanol to recover the FOG from the tea brew. Considering the difference of polarity of catechins and FOG, we add one more step of elution to separate them. After trying a series of the organic solvents, 60% methanol was selected as the best solution.

Table 1 shows that FOG might be effectively recovered with 60% methanol, and about 96% absorbance was collected in the solution. The HPLC–MS test had proved that all the FOG are in this part and the other two parts didn't contain any FOG compounds. Although the absorbance at 287 nm is not exclusively due to catechins, we could deduce when catechins were washed out based on their absorbance spectra and their retention time on HPLC. From Table 1 it can be withdrawn that most catechins were eluted by 100% methanol and not by 60% methanol, so that FOG compounds could be separated from most catechins by this step. This was also confirmed by analyzing catechins by HPLC in these eluates.

### 3.3. Identification of FOG from tea samples on UHPLC and HPLC

Based on the previous mass of experimental data on HPLC and FOG compounds isolation assays by our group since the 1990s (Engelhardt et al., 1992; Hilal, 2010), 18 kinds of FOG compounds were verified and identified from green tea and oolong tea samples this time. The FOG compounds were numbered in the HPLC and UHPLC chromatograms in Fig. 2, based on their HPLC–MS<sup>n</sup>



**Fig. 2.** UHPLC and HPLC separation of green tea FOG compounds. Chromatogram at 354 nm. (above) HPLC; (below) UHPLC. 1, M-rdg; 2, M-rut; 3, M-gal; 4, M-glu; 5, Q-grg; 6, Q-rdglu; 7, Q-rrg; 8, Q-rgal; 9, Q-rut; 10, K-grg; 11, Q-gal; 12, Q-glu; 13, K-drgal; 14, K-rdg; 15, K-gal; 16, K-drglu; 17, K-rut; 18, K-glu.

fragment ions data together with ultraviolet absorbance and retention time.

C<sub>18</sub>-columns were used both on UHPLC and on HPLC. The HPLC method was used for many years in the lab. Now, we tried some new conditions with different column temperature and solvent flow rate, and found that 40 °C was better suited than 20 °C. Comparing with the HPLC method at 20 °C, the better resolution at 40 °C on UHPLC resulted in sharp peaks in the UHPLC chromatogram for the FOG compounds. Furthermore, rutin and K-grg were separated on UHPLC under the selected condition, while there was no separation on HPLC. The contents of these two compounds were really different in different teas, and green tea samples contained more K-grg while oolong tea and black tea had much more rutin. The separation of these two compounds was further confirmed with the addition of rutin solution to both green tea and oolong tea samples. It is not clear whether or not this different content is due to clone, geographic origin or manufacture or to a combination of all factors.

Other FOG compounds, such as acylated kaempferol glycosides could be detected on the corresponding mass tracks, however, the

concentration is much lower and not suitable to be quantified with the current method. This is in principal in tune with the results of Zhao et al., 2011.

The higher column temperature on UHPLC could not only isolate rutin and K-grg, but also change the other compounds' retention time. Comparing the UHPLC chromatogram with the HPLC chromatogram in Fig. 2, we could find that the elution order for M-rut (No. 2) was delayed while Q-gal (No. 11) was ahead. It was concluded from their peak area and related retention time, but was not testified due to lack of standards. This might be caused by the high temperature in UHPLC C<sub>18</sub> column. From the HPLC theory, column temperature could affect both the retention ( $e$ ) and control of selectivity ( $\alpha$ ) and could be used for the optimizing the resolution of compounds on HPLC (Dolan, 2002). Obviously, the retention of these FOG compounds was changed due to the higher 40 °C column temperature, and some other FOG compounds were also affected without changing the elution order, such as compounds No. 15 and 16.

### 3.4. Determination of FOG in green tea, oolong tea and black tea samples by UHPLC vs. HPLC

Based on the identifications of FOG compounds on the UHPLC and HPLC method, we analyzed 3 green tea, 2 oolong tea samples and 3 black tea samples. The total amounts of the FOG in these teas were 2.32–5.67 g/kg dry weight, and there is no significant difference for the total FOG content between green tea, oolong tea and black tea (Table 2). The highest was one black tea sample KEBT from Kenya, but one green tea sample SBXT from China had the similar content, and one oolong tea sample FSOT from China had a little less content. However, we could find that the lowest was from one green tea sample LSYW from China, and one black tea sample ASBT from India had a lower content. It might show that the big difference of FOG content could be sourced from the producing area, and could be affected from some related factors like tea cultivars, soil, rainfall and local climate. The small difference between 2 oolong tea samples of DAOT and FSOT from the same area in Fujian, China, might show that processing could also influence the FOG content in some way.

The proportion of the corresponding aglycones was also investigated. Table 3 shows that there are much kaempferol glycosides in green teas, more quercetin glycosides and myricetin glycosides in oolong teas, while quercetin glycosides dominate in all 3 black

**Table 2**

The comparison of the determination of FOG compounds from green tea and oolong tea samples by UHPLC vs. HPLC (mg/kg).

Method	Green tea			Oolong tea		Black tea		
	WLLJ	LSYW	SBXT	DAOT	FSOT	YNBT	ASBT	KEBT
UHPLC	3679	2334	5673	4270	5021	4657	3005	5597
HPLC	3753	2328	5079	3885	4775	4566	3068	5558

Note: WLLJ, West Lake Longjing green tea; LSYW, Lushan Yunwu green tea; SBXT, Sanbeixiang green tea; DAOT, Dense aroma oolong tea; FSOT, faint scent oolong tea; YNBT, Yunnan black tea; ASBT, Assam black tea; KEBT, Kenya black tea.

**Table 3**

The percentage of three aglycone types of FOG in green tea and oolong tea samples by UHPLC (%).

FOG aglycones	Green tea			Oolong tea		Black tea		
	WLLJ	LSYW	SBXT	DAOT	FSOT	YNBT	ASBT	KEBT
Myricetin	23.4	17.0	17.1	31.7	35.9	3.6	12.5	8.1
Quercetin	23.8	18.3	38.0	52.7	50.8	71.2	65.5	54.1
Kaempferol	52.8	64.7	44.9	15.6	13.3	25.1	22.1	37.7

For abbreviations, see Table 2.

**Table 4**

The content of FOG compounds from green tea and oolong tea samples by UHPLC (mg/kg).

No.	FOG compounds	Green tea			Oolong tea		Black tea		
		WLLJ	LSYW	SBXT	DAOT	FSOT	YNBT	ASBT	KEBT
1	M-rdg	46	38	89	22	17	4	0	0
2	M-rut	51	35	96	244	363	28	0	0
3	M-gal	606	212	457	440	541	52	95	129
4	M-glu	158	113	326	647	883	85	280	326
5	Q-rdglu	260	94	378	159	163	33	11	0
6	Q-grg	138	124	787	759	844	51	32	19
7	Q-rrg	58	17	56	245	189	34	174	0
8	Q-rgal	36	19	230	269	249	377	193	207
9	Q-rut	74	60	263	479	688	1228	406	771
10	K-grg	981	456	639	110	95	0	0	0
11	Q-gal	277	76	310	173	212	474	345	623
12	Q-glu	33	37	132	165	204	1121	805	1410
13	K-drgal	23	29	39	0	0	62	24	0
14	K-rdg	392	612	1183	372	340	79	39	32
15 + 16	K-gal + K-drglu	453	136	307	47	52	86	75	307
17	K-rut	39	135	149	111	149	561	218	702
18	K-glu	56	143	232	28	32	382	307	1072
Total		3679	2334	5673	4270	5021	4657	3005	5597

For abbreviations, see Table 2.



tea samples with more than half of the total aglycone content. The content of all flavonol glycosides is shown in Table 4.

From the results, both black tea and oolong tea samples had the highest content of quercetin types of FOG, and green tea samples had the highest content of kaempferol types of FOG. Referring to the classification of 6 tea classes in the world, both black tea and oolong tea were mostly manufactured from the Large-leaved species and with the process of fermentation, while green tea was mostly manufactured from the Small-leaved species and without the process of fermentation, tea cultivar and processing method might be the uppermost factors for the FOG content in tea samples. This should be further studied.

#### 4. Conclusion

An UHPLC method for the determination of tea flavonol glycosides (FOG) has been developed for the first time, and the tea sample clean-up method by means of polyamide column chromatography was optimized with 3 steps of elutions. With this new UHPLC method, rutin (quercetin-3-O-rutinoside) and K-grg (kaempferol-3-O-glucorhamnoglycoside) were successfully separated. Another advantage of the UHPLC methods is the shorter separation time.

After analyzing the green tea and oolong tea samples, besides 3 black tea samples from China, India and Kenya, the total amounts of FOG compounds ranged from 2.32 to 5.67 g/kg dry weight (calculated as aglycones). Comparing with their original aglycone types, there are many kaempferol glycosides in green teas, more quercetin glycosides and myricetin glycosides in oolong teas, and dominated quercetin glycosides in black teas. Whether the difference in their aglycones was caused by the tea cultivars or planting area, should be further studied on more tea samples.

It is necessary to set up a protocol for the determination of the most abundant FOG in tea. As long as no mass spectral work is necessary and for routine work the polyamide clean-up is not essential as a separation on HPLC in combination with the detection wavelength of 354 nm is selective enough to ensure good data. The UHPLC method could serve as a basis for a relatively fast standard method for the determination of flavonol glycosides in tea.

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