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Determination of flavonoids by high-performance liquid chromatography and capillary electrophoresis

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Abstract

The compounds of flavonoid, an important group in nature, can prevent coronary heart disease and anticancer by virtue of the characteristics of antioxidation. Nine flavonoids most often seen in grape wine, namely apigenin, baicalein, naringenin, luteolin, hesperetin, galangin, kaempferol, quercetin, and myricetine, were determined by means of high-performance liquid chromatography (HPLC) and capillary zone electrophoresis (CZE) in this work. A successful resolution was obtained from an unusual additive of tetrahydrofuran in mobile phase by HPLC. One notable thing is that the mixture of luteolin and quercetin could be separated for the first time by HPLC. In addition, the better detection limit was still attainable even with the use of tetrahydrofuran. The detection limits of CZE performed in borate buffer were hundreds-fold better than in previous reports. Furthermore, the retention and migration behavior of the analytes studied were discussed. As the result of this study, the elution order of flavone and flavonone was reversed to the contention proposed by Wulf et al. It was predictable from the interaction with tetrahydrofuran. Consequently, the extracts from grape wine with solid-phase extraction were analyzed by developing methods of HPLC and CZE. The obtained recoveries ranged from 90 to 107% and the relative standard deviations were under 6.3%.

Keywords: Wine; Food analysis; Flavonoids

1. Introduction

The flavonoidic constituents of wine are responsible for color, astringency and bitterness. They are a large family of over 4000 ubiquitous secondary plant metabolites, comprising five subclasses, anthocyanins, flavonols, flavones, catechins, and flavonones [1]. Flavonoids are always widely used as remedies because of their spasmolytic, antiphlogistic, antiallergic, and diuretic properties [2]. It is assumed that these functions are brought about by the properties of oxygen radical absorbance capacity [1]. The role of flavonoids is related to their chemical structure and chemical variation is present in the basic structure (hydroxylation, methoxylation), the degree of polymerization and the type of conjugation (glycosylation, malonylation, sulphonation) [3]. Flavonols and flavones are flavonoids of particular importance as they were found to contain antioxidant and free radical scavenging activity in foods [4], and it was also indicated in epidemiological studies that their consumption always assured a reduced risk of cancer and cardiovascular disease [5–7]. So far, analysis of flavonoids has been accomplished by thin-layer chromatography [8–11], gas chromatography [12,13], high-performance liquid chromatography (HPLC) [14–17], and capillary electrophoresis (CE) [18–23]. In general, quercetin is the major flavonoid in grape, but there was no way to separate it from luteolin by HPLC in previous studies. Besides, the detection limit was not enough to quantitate trace flavonoids and these limitations have led to invalid quantification results. Thus, improvement in the separation condition is crucial for obtaining accurate results and better detection limits.

The structures of nine flavonoids studied, frequently found in grape wine, are listed in Table 1, and they are categorized under skeletons of flavanone and flavone. From the viewpoint of biosynthesis, caffeic acid is the potential precursor of flavonoid, and it is always extracted along with flavonoid from acidic media. Thus, it is important to develop an analytical method of these 10 analytes together, caffeic acid accompanied with nine flavonoids. The HPLC and capillary zone electrophoresis (CZE) analytical conditions established in this study were applied to determine the content of flavonoids in real commercial samples of grape wine. Moreover, the retention migration behavior of these compounds is also discussed.

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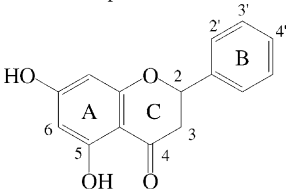
Table 1
List of compounds studied



Flavonoid	C_2–C_3	C_3	C_6	$C_{3'}$	$C_{4'}$	$C_{5'}$
Apigenin (4′,5,7-trihydroxyflavone)	=	H	H	H	OH	H
Baicalein (5,6,7-trihydroxyflavone)	=	H	OH	H	H	H
Luteolin (3′,4′,5,7-tetrahydroxyflavone)	=	H	H	OH	OH	H
Naringenin (4′,5,7-trihydroxyflavanone)	–	H	H	H	OH	H
Hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone)	–	H	H	OH	OMe	H
Galangin (3,5,7-trihydroxyflanone)	=	OH	H	H	H	H
Kaempferol (3,4′,5,7-tetrahydroxyflavone)	=	OH	H	H	OH	H
Quercetin (3,3′,4′,5,7-pentahydroxyflavone)	=	OH	H	OH	OH	H
Myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone)	=	OH	H	OH	OH	OH

2. Experimental

2.1. Chemicals

Apigenin (4′,5,7-trihydroxyflavone) and galangin (3,5,7-trihydroxyflanone) were obtained from Aldrich (USA). Baicalein (5,6,7-trihydroxyflavone), naringenin (4′,5,7-trihydroxyflavanone) and hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone) were purchased from Lancaster (UK). Luteolin (3′,4′,5,7-tetrahydroxyflavone), quercetin (3, 3',4',5,7-pentahydroxyflavone) and caffeic acid (3,4-dihydroxycinnamic acid) were brought from Sigma (USA). Kaempferol (3,4′,5,7-tetrahydroxyflavone) was obtained from TCI (Japan) and myricetine (3,3′,4′,5,5′,7-hexahydroxyflavone) from Fluka (Switzerland). The purities of the 10 analytes were up to 98%. The solvent of tetrahydrofuran (THF) and acetonitrile, labeled as HPLC grade, accompanied with sodium tetraborate (borax) and C_{18} solid-phase extraction (SPE) cartridge were purchased from Merck (Germany). Grape wine was collected from retail stores locally.

2.2. Instrumentation

HPLC apparatus consisted of Perkin-Elmer 1020 LC plus integrator linked to a model 235 C diode-array detection (DAD) system, 250 B delivery pump and an injection valve with a 100 μl sample loop. The wavelength was set at 270 nm. The separations were performed on a Hypersil BDS C_{18} column (5 μm, 250 mm × 4.6 mm i.d.). All CZE separations were performed with a SpectraPhoresis 100 capillary electrophoresis system from Thermo Separation Products TSP (Fremont, CA, USA). The fused silica capillary was deactivated and obtained from J&W (USA). Signal was monitored at 250 nm with a UV–Vis SC-100 detector purchased from TSP. Electropherograms were recorded with a Maxima 820 chromatography workstation from Scientific Information Service Corporation (Taipei, Taiwan).

2.3. Methodology

The optimum separation of HPLC was eluted by 30% acetonitrile for 8 min with flow rate of 1.2 ml/min, then gradient the acetonitrile into 100% in 3 min, and kept for 4 min. The aqueous solution of mobile phase was prepared by 1% aqueous THF and adjusted to pH 3 with phosphoric acid.

The optimum separation condition of CZE was performed by 35 mM borax, pH 8.9, and applied 240 V/cm field strength on a fused capillary of 70 cm (effective length: 45 cm) × 75 μm. Capillary tube was conditioned prior to use by 1 M of NaOH for 20 min and deionized water for 60 min. Further equilibrium was performed with 1 M NaOH for 2 min, deionized water for 2 min and corresponding run buffer for 2 min. All the samples including standard solutions were filtered through a 0.45 μm syringe filter. Standards and samples were injected into the capillary by vacuum injection at a fixed time of 1.0 s.

2.4. Sample preparation

The grape wine sample 20.00 ml was concentrated to dryness by vacuum rotatory. The residual was dissolved in 3 ml of pH 2.5 hydrochloric acid, and then passed through an SPE cartridge. The interference matrix was eluted with 8 ml of 20% acetonitrile aqueous solution of pH 2.5. Then, the extract was collected from the developing elution with 3 ml acetonitrile, and finally filtered through a 0.45 μm syringe filter before analysis by HPLC and CZE.

3. Results and discussion

3.1. High-performance liquid chromatography

In general, the pK_a of flavonoid with different positions of hydroxyl group are ranged from 7–12. In slight acidic

medium, caffeic acid acted as an anionic particle due to pK_{a1} of 4.64 and pK_{a2} of 9.45. Hence, the pH of mobile phase was the first thing to be ascertained. To maintain caffeic acid in neutral particle, we controlled the aqueous at pH of 3 for the separation. Luteolin and quercetin were not completely dissolved probably because of similar interaction with column caused by similar chemical properties. Naringenin and kaempferol were also difficult to be distinguished. Therefore, effects of some additives to the solvent were examined. THF showed the best result among some commonly used additives, tributylamine, β-cyclodextrin, sodium dodecyl sulfate and cetyl trimethyl ammonium bromide. This was consistent to our experience that addition of little amount of THF always gave a better separation of aromatic compounds than other additives. Next, concentration of THF was optimized. Fig. 1 displayed the result of THF additive from 0 to 3%. As a result, 1% of THF as additive to the mobile phase was chosen. After optimizing the composition of mobile phase and the follow-up step for gradient elution, the optimum mobile phase consisted of 30% acetonitrile and 70% of 1% aqueous THF, pH 3, with flow rate of 1.2 ml/min for 8 min, and then gradient the acetonitrile into 100% in 3 min, and kept for 4 min.

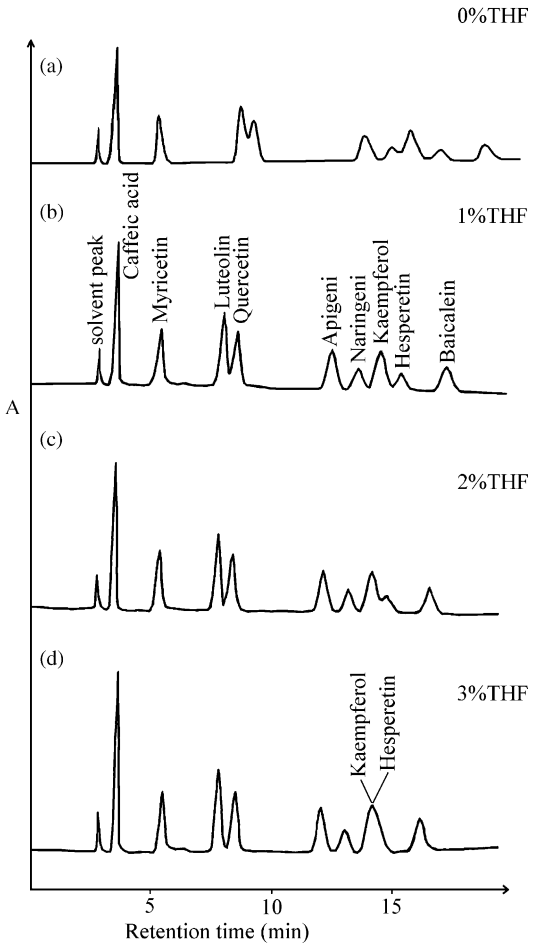


Fig. 1. The chromatograms obtained with various percentage of: (a) 0%, (b) 1%, (c) 2%, and (d) 3% THF. Conditions as in Section 2.3.

Even with identical skeleton, the value of the capacity factor (k') varies with the different position of hydroxyl group. From the previous report by Wulf and Nagel [14], the larger electron density will make the hydrogen bond between the C_5–hydroxyl group and the C_4–keto group stronger and make both functional groups appear less polar to the solvent. In flavanones, the C_4–keto group may be out of plane of the adjacent phloroglucinal ring (called as C-ring), thus making a hydrogen bond with the *peri*-hydroxyl group weaker and expose both functionalities to stronger interactions with the solvent. According to what has been mentioned above, Wulf and Nagel [14] inferred that flavones, because of their total planarity, simply might be more difficult to solvate than the partially planar flavanones, and become less polar than flavanones. But, the result appeared to be on the contrary in this study. We obtained a reverse result from apigenin and narigenin. This evidence was proved by comparison of the capacity factor between apigenin and naringenin. Although apigenin possesses a double bond at C_2, it moves faster than naringenin, that is to say the k' value of apigenin is smaller than naringenin. We assumed confidently that the influence was caused by THF. It could be the only possible explanation for this abnormal behavior. The k' values of studied compounds are listed in Table 2. With regard to the influence of flavonoids without hydroxyl group at B-ring on linear relationship, baicalein and galangin showed poor linearity over the concentration of 10.0 μg/ml and rather remained on column. We speculated that it was induced by poor solvation with mobile phase consisting of aqueous THF–acetonitrile. Besides, we found that the flavonoid with one substitution of hydroxyl group at C-ring made the value of k' increase from the comparison between apigenin and kaempferol, and luteolin and quercetin. Moreover, the polarity is different from the substitution of hydroxy group at various positions. From the comparison between kaempferol and luteolin, galangin and apigenin, galangin and baicalein, we made two points. The first one was the substitution of hydroxyl group at C-ring (C_3), which generated more polar than at B-ring. And the

Table 2
Reproducibility and k' value of the studied flavonoids of HPLC

Compounds	R.S.D.[a](%)				k'
	Retention time		Integrated area		
	Run-to-run	Day-to-day	Run-to-run	Day-to-day	
Caffeic acid	0.34	0.38	2.28	1.58	
Myricetin	0.53	1.00	3.82	2.98	0.62
Luteolin	0.35	1.98	1.87	0.76	1.67
Quercetin	0.45	2.24	3.10	1.18	1.92
Apigenin	0.44	2.73	1.42	1.81	3.23
Naringenin	0.36	2.77	1.39	2.21	3.61
Kaempferol	0.46	2.54	0.70	2.23	3.87
Hesperetin	0.28	0.73	1.55	1.60	4.13
Baicalein	0.10	0.09	1.99	0.78	4.25
Galangin	0.31	0.25	2.28	3.07	4.71

[a] The result was obtained from 10.00 μg/ml with six measurements and the analytical conditions were as in Section 2.2.

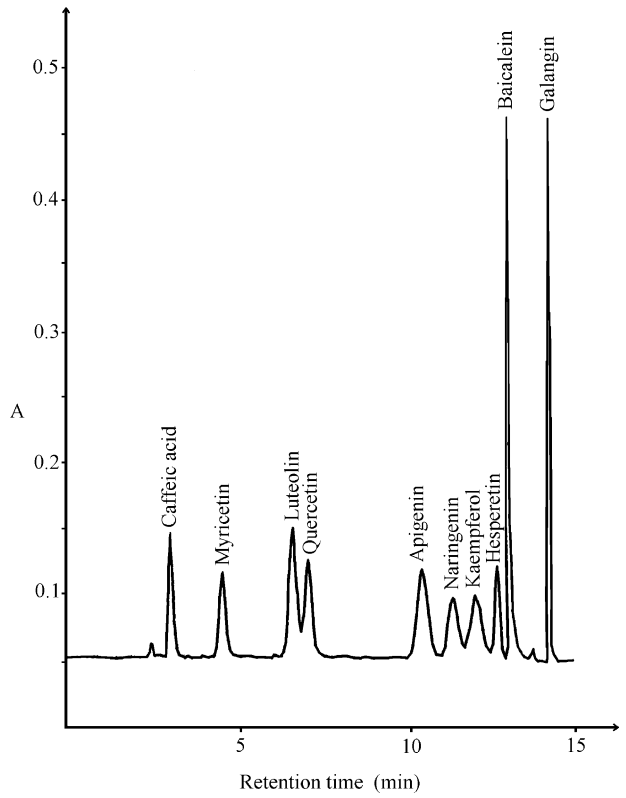


Fig. 2. The optimum chromatogram of the flavnoids. Conditions as in Section 2.3.

second was the substitution of hydroxyl group at C-ring (C_3), which had a greater influence in generating more polar than at C_6 of A-ring. Furthermore, the method developed in this study was the first to successfully separate the mixture of luteolin and quercetin by HPLC.

The chromatogram under optimized condition is shown as Fig. 2. The appropriate k' and good reproducibility, with R.S.D. 4%, for five measurements were listed in Table 2. Other results, such as detection limit, linear range, calibration curve, correlation coefficient and theoretical plates, are listed in Table 3. The detection limit was obtained by three times of standard deviation though dividing the slope; meanwhile, the standard deviation was calculated from six measurements of 10.0 μ/ml of standard solution by run-to-run and day-to-day.

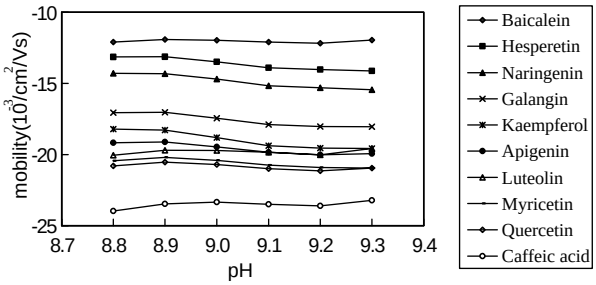


Fig. 3. The effect of pH on electrophoretic mobility.

3.2. Capillary zone electrophoresis

The key factor of separation in capillary zone electrophoresis, the simplest mode of capillary electrophoresis, is based on the charge-to-mass ratio. The charge amount of particle varies with the electrolyte buffer of pH. In other words, the pH of buffer is the major factor for charge amount. For monohydroxyflavone, the pK_a of C_7-hydroxyl group is 7.39 [24]. The pK_a of studied compounds should be completely or partially ionized with pH higher than 8. We presumed confidently that all of the solutes studied are charged particles with pH above 8. Thus, we investigated the various pH from 8.0 up to 10.0. The obvious variations ranged from 8.8 to 9.3 and the result was shown in Fig. 3. The electrolyte used in this work was borax. The effect of the concentrations of borax buffer on migration time and resolution were shown in Table 4. Current increased with the ionic strength of buffer, and the Joule heat increased as well. Furthermore, the migration time increased with ionic strength. Five concentrations from 30 to 50 mM with 5 mM increment were studied. In the comparison of the resolution, 35 mM was adopted as the better resolution of 1.25 and shorter analytical time of 18 min. The driving force behind the migration of ions in CZE is the electric field strength applied across the capillary, which is related to the applied voltage (V) over the total capillary length (cm). Both the electrophoretic migration velocity and electroosmotic flow

Table 3
Quantitative results of studied flavonoid by HPLC

Compound	Detection limit (μg/ml)	Linear range (μg/ml)	Linearity	Correlation coefficient	Theoretical plates ($\times 10^{-3}$)
Caffeic acid	0.09	0.10–60	$y = 1.71x + 0.52$	0.9995	1.25
Myricetin	0.16	0.30–80	$y = 1.84x - 0.19$	0.9990	1.54
Luteolin	0.09	0.10–60	$y = 2.56x + 0.35$	0.9999	3.85
Quercetin	0.14	0.10–60	$y = 2.08x + 0.57$	0.9996	3.07
Apigenin	0.16	0.30–70	$y = 2.61x + 0.61$	0.9997	4.93
Naringenin	0.21	0.30–70	$y = 1.69x + 0.64$	0.9993	4.51
Kaempferol	0.13	0.10–80	$y = 2.35x + 0.25$	0.9999	5.06
Hesperetin	0.11	0.10–70	$y = 1.46x + 0.41$	0.9997	39.3
Baicalein	0.08	0.10–10	$y = 4.06x + 0.59$	0.9994	92.8
Galangin	0.17	0.30–10	$y = 3.06x + 0.31$	0.9995	136

Table 4
Influence of borate concentration on migration time and resolution in CZE[a]

Compound	Migration time[b] (min)				
	Borate concentration (mM)				
	30	35	40	45	50
Baicalein	8.6	9.1	9.2	9.3	9.5
Hesperetin	9.0	9.6	9.8	10.0	10.2
Naringenin	9.5	10.1	10.3	10.6	10.9
Galangin	10.8	11.7	12.0	12.4	12.8
Kaempferol	11.5	12.5	12.9	13.4	14.0
Apigenin	12.0	12.8	13.5	14.0	14.7
Luteolin	12.7	13.6	14.0	14.4	15.1
Myricetin	12.9	14.1	14.6	15.2	15.9
Quercetin	13.2	14.5	15.0	15.6	16.3
Caffeic acid	16.1	18.0	18.9	19.9	21.3
R_s[c]	1.09	1.25	1.32	1.43	1.57

[a] The condition was performed by pH 8.9, 70 cm (effective length: 45 cm) × 75 μm capillary, applied voltage 15.4 kV, and detection wavelength 250 nm.
[b] $n = 3$.
[c] R_s means the resolution of myricetin and quercetin.

velocity are in direct proportion to the electric field. In general, excessive field strength will result in a shorter analysis time, but the Joule heat production limits the application of high field strength. Therefore, higher efficiency and better resolution will be obtained by working with field strength as high as possible. In this study, the capillary length was fixed at 70 cm (45 cm effective length) and the field strengths were various from 220 to 260 V/cm. The optimum was achieved at 240 V/cm, applied voltage 16.8 kV, with an analysis time of within 16 min. The resulted electropherogram under the optimum operation condition with resolution of 1.21 of myricetin and quercetin is shown in Fig. 4.

The reproducibility of migration time and integrated area was determined for run-to run and day-to-day with 5.0 μg/ml

Table 5
Reproducibility of flavonoids in CZE[a]

Compound	R.S.D.[b] (%)			
	Integrated area		Retention time	
	Run-to-run	Day-to-day	Run-to-run	Day-to-day
Baicalein	0.79	2.41	0.24	1.68
Hesperetin	2.89	2.42	0.27	1.73
Naringenin	1.83	5.39	0.32	1.73
Galangin	2.75	5.95	0.34	1.84
Kaempferol	3.91	5.88	0.32	1.79
Apigenin	3.81	5.04	0.30	1.93
Luteolin	3.18	1.53	0.30	0.73
Myricetin	2.93	0.39	0.30	0.58
Quercetin	2.11	0.42	0.29	1.01
Caffeic acid	1.39	1.33	0.39	2.81

[a] The condition was performed by 35 mM of borate buffer, pH 8.9, 70 cm (effective length 45 cm) × 75 μm capillary, and electric field strength of 240 V/cm.
[b] $n = 6$.

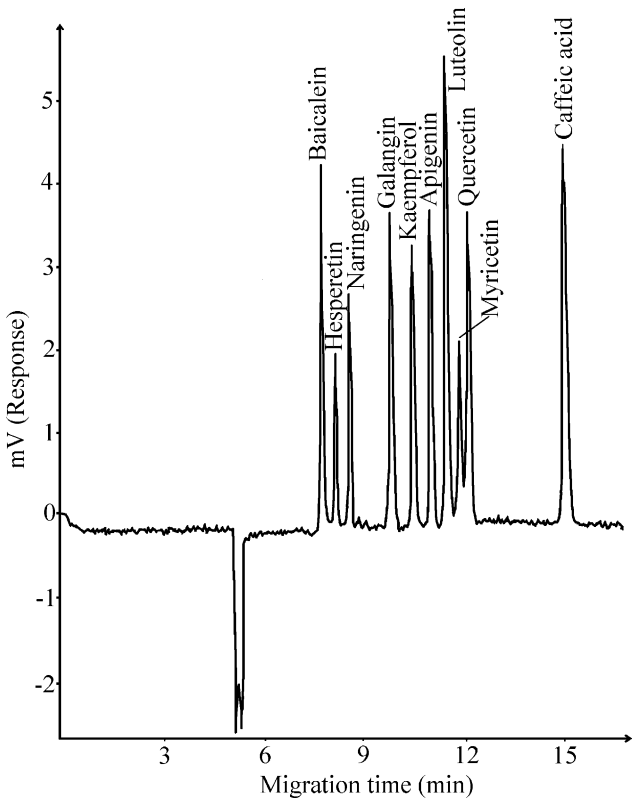


Fig. 4. The optimum electropherogram of the flavonoids. Conditions as in Section 2.3.

of each solute. The results were shown in Table 5. All R.S.D.s were under 6% of integrated area for day-to-day with six measurements. The results of detection limit, linear range, correlation coefficient, and linearity were shown in Table 6. The linear range of the analysis was studied by series of injections of standard mixture containing various concentrations. The linear ranges and detection limits were obtained with the same method as HPLC. The obtained detection limits in this study were better than those in previous papers for hundreds-fold [21,22].

From the above results, we can draw a conclusion about the different position of substituents on migration factor. Flavones with hydroxyl group at $C_{4'}$, such as apigenin, luteolin, kaempferol, quercetin, myricetin, are faster in mobility, and longer in migration time due to the larger charge amount and superior extent of partial or complete ionization. To compare the mobility of apigenin and galangin, apigenin is faster because the pK_a of hydroxyl group at $C_{4'}$ is lower than at C_3. From the evident results, apigenin was faster in mobility than narigenin because a double bond at C_2 of apigenin is the only difference from narigenin. We assumed that promoting the protonic ionization of hydroxy at $C_{4'}$ in flavone of apegenin was caused by the conjugation in C-ring. Moreover, we found a meaningful inference from the migration pattern in the order of baicalein, ganangin, and apigenin. The hydroxy group at different position, at C_6, C_3, and $C_{4'}$, represented the extent of protonic

Table 6
Quantitative results of flavonoids by CZE[a]

Compound	Detection limit (μg/ml)	Linear range (μg/ml)	Linearity	Correlation coefficient	Theoretical plates ($\times 10^{-3}$)
Baicalein	0.050	0.050–30.0	$y = 4053x + 397$	0.9991	64.9
Hesperetin	0.150	0.100–30.0	$y = 1657x + 341$	0.9992	103
Naringenin	0.100	0.100–150	$y = 2384x + 341$	0.9995	79.3
Galangin	0.210	0.300–20.0	$y = 4261x + 353$	0.9998	37.6
Kaempferol	0.080	0.100–140	$y = 4369x - 364$	0.9993	52.9
Apigenin	0.090	0.100–70.0	$y = 4673x + 114$	0.9996	47.3
Luteolin	0.050	0.050–90.0	$y = 7160x + 157$	0.9994	51.5
Myricetin	0.600	1.000–30.0	$y = 2433x - 820$	0.9990	54.8
Quercetin	0.060	0.100–60.0	$y = 5155x - 75$	0.9991	57.4
Caffeic acid	0.030	0.050–30.0	$y = 7773x - 9$	0.9995	56.3

[a] The applied conditions were described in Section 2.3.

Table 7
The flavonoids content in grape wine determined by HPLC and CZE

Compound	Found (μg/ml)[a]				Recovery (%) (R.S.D. (%))		Content in sample (μg/ml)	
	HPLC		CE		HPLC	CE	HPLC	CE
	Direct	Spike[b]	Direct	Spike[b]				
Myricetin	1.57	11.07	0.96	9.97	94.8 (6.27)	90.1 (5.68)	0.39	0.24
Luteolin	–[c]	–[c]	ND	9.34	95.4 (2.02)	93.4 (1.69)	–[c]	ND
Quercetin	9.93	19.80	8.92	18.18	98.7 (0.48)	92.6 (0.43)	2.48	2.23
Apigenin	ND	10.17	ND	9.69	102 (0.56)	96.9 (0.39)	ND	ND
Naringenin	0.54	10.88	–[d]	–[d]	103 (4.10)	99.8 (5.23)	0.14	–[d]
Kaempferol	1.83	11.95	1.02	10.08	101 (1.72)	90.6 (3.73)	0.46	0.27
Hesperetin	0.31	10.98	0.47	9.85	107 (1.84)	93.8 (4.19)	0.08	0.12
Baicalein	1.21	11.19	0.82	10.35	99.8 (5.43)	95.3 (4.68)	0.30	0.21
Galangin	ND	9.43	–[d]	–[d]	94.3 (1.24)	92.7 (3.56)	ND	–[d]

[a] The determined value was obtain from concentrated sample for four-folds with three measurements.
[b] The spiked concentration was 10 μg/ml.
[c] It was identified as impurity, not luteolin, with standard of λ_{max}, PI value and ratio value.
[d] Noise of sample matrix was present.

ionization of hydroxy group was in the order of $C_{4'}$, C_3, and C_6. It also agreed with what has been discussed above.

3.3. Quantitative of flavonoids in grape wine

The extracts from grape wine with solid-phase extraction were analyzed by developed HPLC and CZE with direct and spiked methods. The content and recovery are shown in Table 7. The obtained acceptable recoveries ranged from 90 to 107% and R.S.D.s were under 6.3% with three extracts. The major constituent of flavonoid in grape wine is quercetin, the minors are kaempferol, myricetin, baicalein, naringenin, which are always along with trace amount of hesperetin.

4. Conclusion

In summary, the methods developed in this study were successfully applied to the analysis of commercial grape wine, and accurate results were obtained as a consequence. Although efforts of separating some of the analytes had been made in previous papers, to separate the mixture of luteolin and quercetin successfully was definitely the first time. The importance of this study lies in the better detection limit and shorter time consumption. Furthermore, we discussed particularly the retention behavior in HPLC and migration behavior in CZE from their polarity and pK_a. Even though the obtained elution was in reverse order to Wulf's inference, we speculated confidently that it was caused by the interaction of THF.

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