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Article

Ultra-Performance Liquid Chromatography for the Simultaneous Quantification of Rutin and Chlorogenic Acid in Leaves of *Ribes* L. Species by Conventional and Chemometric Calibration Approaches

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A new ultra-performance liquid chromatography (UPLC) using conventional and chemometric calibrations was improved for the simultaneous estimation of chlorogenic acid (CA) and rutin (RUT) in leaves of *Ribes* L. species (*R. rubrum*, *R. biebersteinii*, *R. nigrum*, *R. uva-crispa*, *R. alpinum*, *R. orientale*, *R. multiflorum* and *R. anatolica*). The UPLC separation for CA and RUT in samples were performed on a Waters UPLC BEH phenyl column (100 mm × 1.0 mm i.d., 1.7 μm) and mobile phase consisting of acetonitrile and 0.1 M formic acid buffer, pH = 3.77 (15:85, v/v) containing 1.0 mL triethylamine in 1,000 mL mobile phase. Multi-wavelength UPLC chromatograms of CA and RUT in calibration and plant samples were obtained by the photodiode array (PDA) detection at the wavelength set from 290 to 360 nm with the interval of $\Delta\lambda = 10$ nm. Conventional UPLC-single and chemometric calibrations were subjected to the analysis of the related compounds. By using UPLC data, conventional and chemometric calibrations in the linear concentration range between 2.5 and 40.0 μg/mL for all compounds were applied for the simultaneous quantitative analysis of CA and RUT in plant samples of seven different *Ribes* species.

Introduction

As it is expressed in the systematic classification, *Ribes* species belong to the Grossulariaceae family. This family only consists of *Ribes* genus and is especially common in the north temperate zone of the world. *Ribes* genus contains about 200 taxa all over in the world. Some *Ribes* species are very important berry from commercial point of view. In addition, some of the *Ribes* species have been used as ornament (1). The leaves of species have diuretic and antirheumatic properties, which have been being used in traditional medicine in the world (2, 3).

In the presented work, *R. rubrum* L. (cultured), *R. biebersteinii* Berl. ex. DC., *R. nigrum* L., *R. uva-crispa* L., *R. alpinum* L., *R. orientale* Desf., *R. multiflorum* Kit. ex Romer&Schultes and *R. anatolica* Behçet (Endemic) grown in Turkey were considered (4–6). Particularly, leaves of *R. nigrum*, *R. rubrum* and *R. orientale* have been utilized in folk medicine in Turkey due to their diuretic and diaphoretic properties (7).

In general, chemical and biological activities of berries of *Ribes* species have been investigated. Leaves of *Ribes* species commonly contain phenolic compounds, especially flavonoid and phenolic acids (8–15). Also, the content of leaves of *Ribes* species in essential oils has been analyzed (16, 17). Biological activities such as antioxidant (18–23), anti-inflammatory (24–27), antihypertensive (28, 29) and antimicrobial (30–34) have been reported on *Ribes* species leaves.

As described above, biological and chemical properties of *Ribes* species are very interesting for traditional treatment. For this reason, the analysis of *Ribes* species is very important task in order to reach more information about their content by applying new powerful analytical methods. A literature survey shows that several conventional chromatographic methods were reported for the analysis of CA and RUT in *Ribes* leaves (9, 11, 14). However, there is no conventional UPLC-single or -multivariate calibration method reported for the simultaneous analysis of CA and RUT in their plant samples.

In the application of the conventional HPLC or UPLC-single calibration, the selection of the detection wavelength is an important problem for the simultaneous separation of different compounds, especially when the analyzed compounds have different maximum absorption wavelengths. For this reason, the optimal wavelength providing a maximum absorption band could be selected for each compound to obtain successful chromatographic measurements. Otherwise, conventional HPLC or UPLC method based on the unsuitable single-wavelength detection may not provide us expected analysis result for multicomponent determination. In addition, chromatographic peak fluctuation can produce unsuitable chromatographic peaks such as distorted peaks, baseline drift and change in peak areas from injection to injection during the online data collection in an analytical instrument. If the single-wavelength detection is used for HPLC or UPLC calibration, all information related to chromatographic peak can be lost because of the mentioned fluctuation and bad baseline, etc. In such case, a good calibration is not possible to get successful analysis results. All of them have an effect on the precision and accuracy of the determination results. The use of multi-wavelength UPLC combined with multivariate calibration such as PCR and PLS, etc. enables to overcome the mentioned disadvantages of the conventional single calibration chromatographic approaches (35, 36). Thus, the combined use of UPLC and multivariate calibration based on the multivariable chromatographic responses will give us opportunity for increasing the quality, reliability, precision and accuracy of the analysis results.

In this study, conventional UPLC and UPLC-multivariate approaches based on the single-wavelength detection and multi-wavelength detection were developed and applied for the simultaneous determination of CA and RUT in leaves of seven different *Ribes* species in Turkey. In case of the chemometric analyses, the principal component regression (PCR) and partial least squares (PLS) algorithms were subjected to the multi-wavelength UPLC

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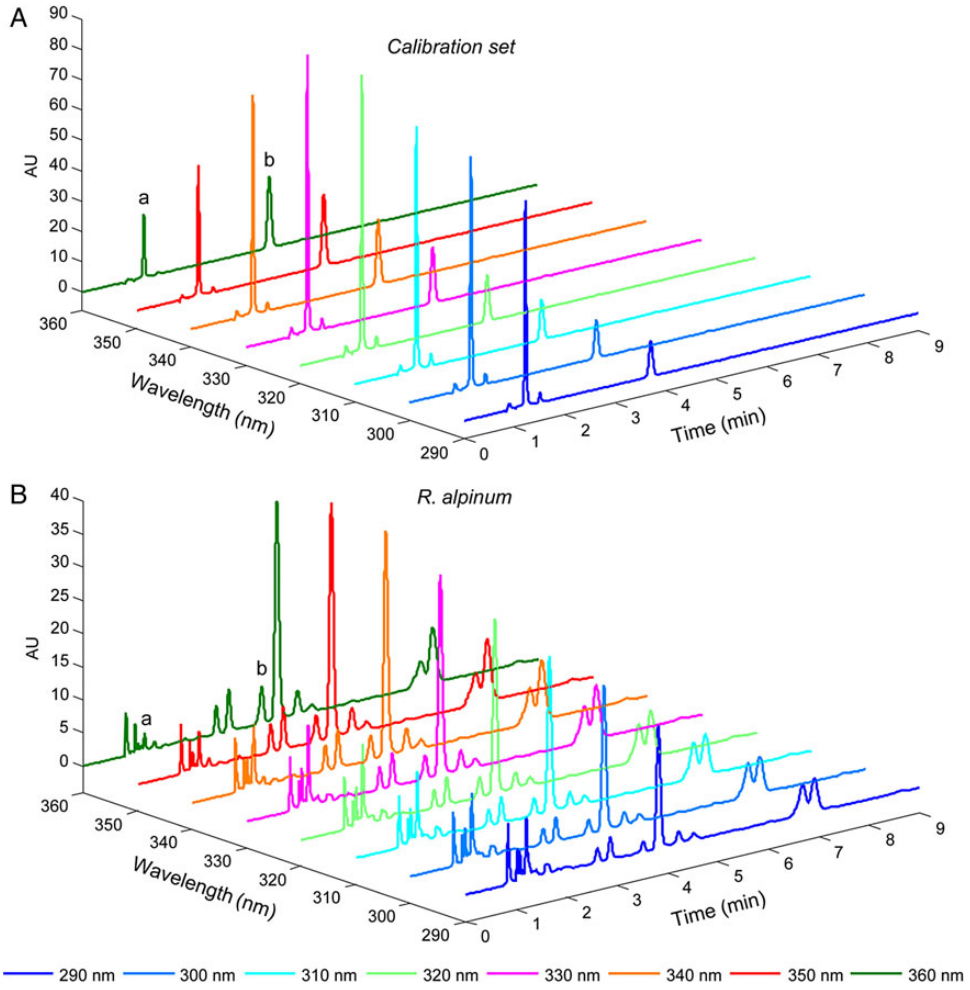


Figure 1. (A) Multi-wavelength UPLC chromatograms of 20 μg/mL CA (a) and 20 μg/mL RUT (b) in the calibration set 5 and (B) multi-wavelength UPLC chromatograms of CA (a) and RUT (b) in *R. alpinum*. This figure is available in black and white in print and in color at *JCS* online.

dataset. Satisfactory results for the analysis of seven different *Ribes* species were reported.

Theoretical background

In analytical chemistry, it is difficult to obtain ideal measurements for chemical and pharmaceutical analyses. Analytical measurements may be affected by experimental interferences coming from instrumentation process as well as by chemical and physical interferences due to phenomena in samples. PCR and PLS algorithms are very powerful tool to remove the effects of some experimental interferences and instrumentation errors. In addition, these multivariate calibration methods are used to obtain highest precise and accurate results of the determination of compounds in complex multicomponent samples.

UPLC-PCR approach

In the UPLC-PCR approach, the peak area (R, which corresponds to chromatographic response) of the calibration set (C, which corresponds to the concentration set) of the related compounds are processed by mean-centering procedure giving R_o and C_o, respectively. The variance–covariance dispersion matrix of the centered matrix R_o is computed. The normalized eigenvalues and eigenvectors are obtained starting from square variance–covariance matrix. The number of the optimal principal components, which correspond to eigenvectors (P), is selected by considering only the highest values of the eigenvalues providing low standard error of calibration (SEC) and standard error of prediction (SEP). The other eigenvalues and their eigenvectors, which correspond to random errors coming from instrumentation noise and other experimental sources, are eliminated. To accomplish this objective, the UPLC-PCR calibration coefficient (b) is calculated using the equation expressed by $b = P \times q$. Where P is the matrix of eigenvectors and q is the C-loadings given by $q = D \times T^T \times R_o$. T^T is the transpose of the score matrix T, and D is a diagonal matrix, which corresponds to the inverse of the selected eigenvalues. The content of compounds in samples is determined by using the $C_{\text{prediction}} = b \times R_{\text{sample}}$.

UPLC-PLS approach

In the case of the UPLC-PLS, the PLS calibration algorithm involves the independent (C, which corresponds to the

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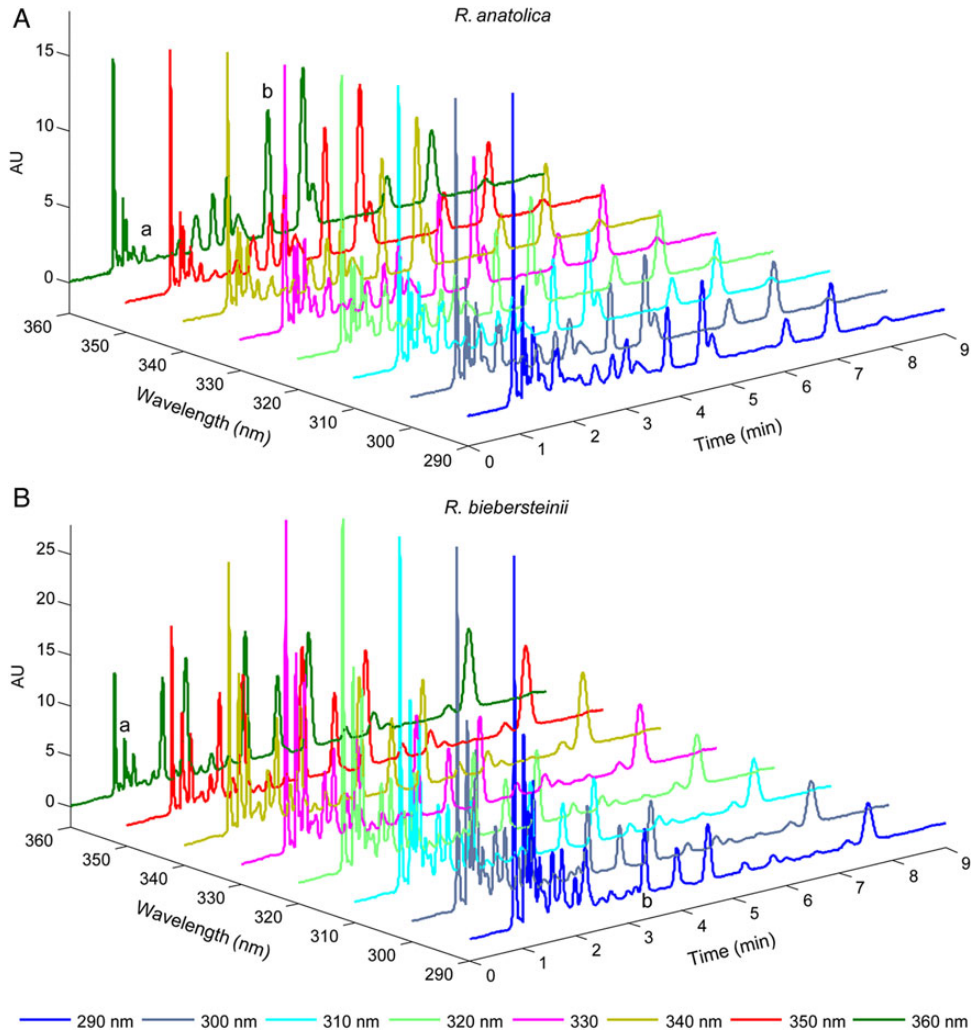


Figure 2. Multi-wavelength UPLC chromatograms of CA (a) and RUT (b) in *R. anatolica* (A) and *R. biebersteinii* (B). This figure is available in black and white in print and in color at *JCS* online.

concentration set) and dependent variables (R, which corresponds to chromatographic response) on the data compression and decomposition operations. In practice, the UPLC-PLS calibration is done by decomposition of both concentration and the peak area matrix into latent variables, $R = T \times P^{T} + E$ and $C = U \times Q^{T} + F$, where R and C are peak area and concentration, respectively; T and U are the scores for R and C, respectively; P^{T} and Q^{T} are the loadings for R and C, respectively; E and F are the residuals for R and C, respectively. The PLS regression equation, $C_{\text{prediction}} = b \times R_{\text{sample}}$, is applied for the quantitative estimation of compounds in samples. The vector, b is calculated using $b = W \times (P^{T} \times W)^{-1} \times Q$, where W is a weight matrix.

Experimental

Instrument and software

UPLC analysis was performed on a Waters Corp. (Milford, MA) ACQUITY UPLC H-Class system, including a quaternary solvent manager, PDA detector, cooling autosampler and oven enabling control of the temperature of the analytical column. Data were collected and processed by Waters® Empower2 software. Chromatographic separation was achieved on a Waters UPLC BEH phenyl column (100 mm × 1.0 mm i.d., 1.7 μm), thermostated at 35°C. PCR and PLS calibrations and related data treatments were computed by means of Matlab 7.12 and Microsoft Excel Softwares.

Chemicals and reagents

Acetonitrile is of HPLC grade (Sigma Aldrich), formic acid (Merck) and trietylamine (Merck) are of reagent grade. Active compounds, rutin (RUT, Sigma R5143) and chlorogenic acid (CA, Sigma C3878) were obtained from Sigma Chemical Industry. Chromatographic grade double-distilled water was used for the preparation of the buffer solutions during chromatographic analysis.

UPLC method development and optimization

Owing to very important medicinal properties of *Ribes* species, we focused mainly on the development of new sensitive,

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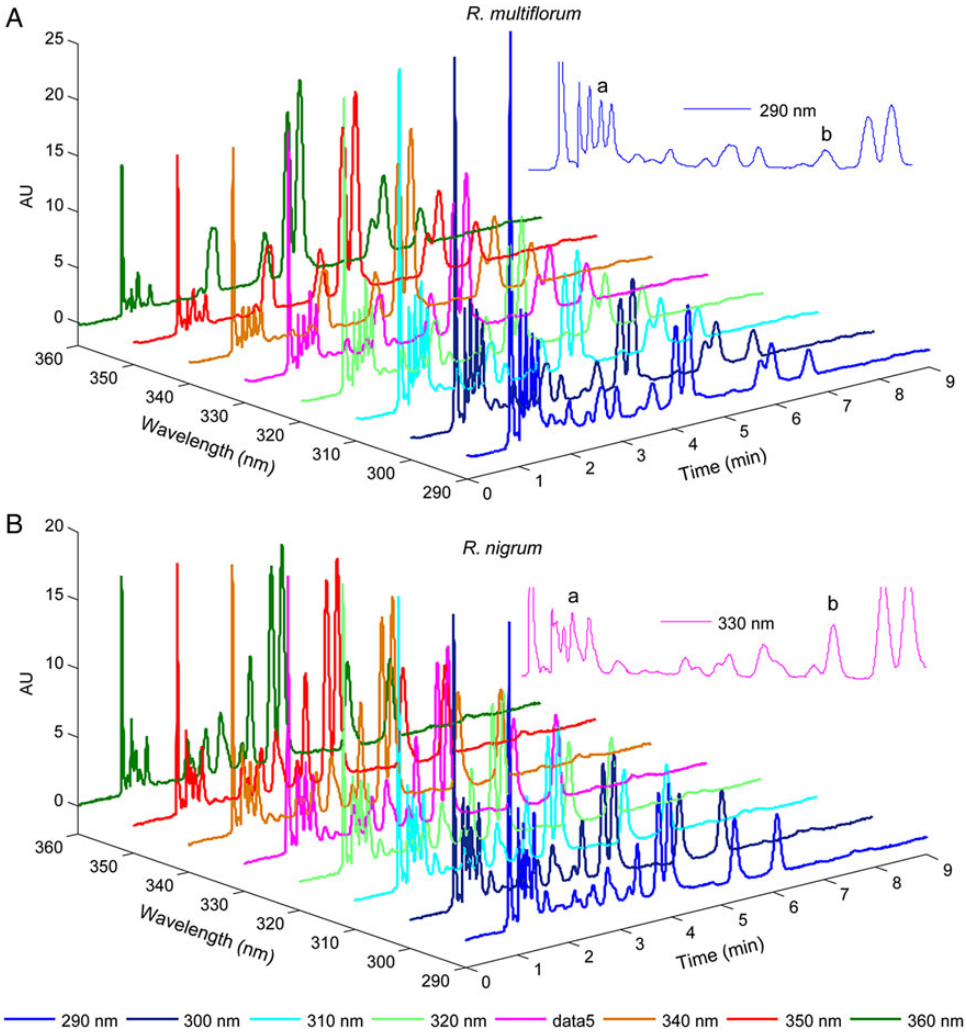


Figure 3. Multi-wavelength UPLC chromatograms of CA (a) and RUT (b) in *R. multiflorum* (A) and *R. nigrum* (B). This figure is available in black and white in print and in color at *JCS* online.

selective, rapid and reliable method for the simultaneous quantitative chromatographic estimation of the amount of CA and RUT in leaves consisting of the mentioned species. For this aim, new conventional UPLC-single and -multivariate calibration (PCR and PLS) methods were improved for the fast quantitative analysis of *R. rubrum*, *R. biebersteinii*, *R. nigrum*, *R. uva-crispa*, *R. alpinum*, *R. multiflorum*, *R. anatolica* containing CA and RUT compounds. Especially, one of *Ribes* species, which has been grown in Turkey is *R. orientale*, but it was not considered because the quantity of CA and RUT in leaves of *R. orientale* can be not detected by applying the UPLC method.

In the preliminary experimental assays, several mobile phases systems with various proportions of organic solvents at different pHs, flow rates and column temperatures were tested to find optimum chromatographic conditions for the determination of RUT and CA by the newly developed conventional UPLC method and UPLC-multivariate calibration approaches (UPLC-PCR and UPLC-PLS). From these studies, the mobile phase consisting of acetonitrile and 0.1 M formic acid buffer, pH = 3.77 (15:85, v/v) in the presence of 1.0 mL triethylamine in 1,000 mL mobile phase and an isocratic flow rate of 0.3 mL/min at column temperature 30°C was found to be very suitable chromatographic conditions for an efficient separation and satisfactory determination of CA and RUT in plant samples. The elution of the mobile phase was done in an isocratic manner through a Waters UPLC BEH phenyl column (100 mm × 1.0 mm i.d., 1.7 μm), followed by UV detection at the wavelength set from 290 to 360 nm. Under these optimal chromatographic conditions, new conventional UPLC approach based on single UV detection was applied for the analysis of *Ribes* species. As a way of the elimination of the random errors coming from instrumentation noises, baseline errors and chromatographic fluctuations, the UPLC-PCR and UPLC-PLS models applied to the multi-wavelength UPLC data were improved for obtaining more desirable analysis results for CA and RUT in same real plant samples to get higher accurate prediction results with low standard deviations or relative standard deviations.

Preparation procedure of plant samples

Aerial parts of all species were collected at different periods of years and from the various locations in Turkey. After that,

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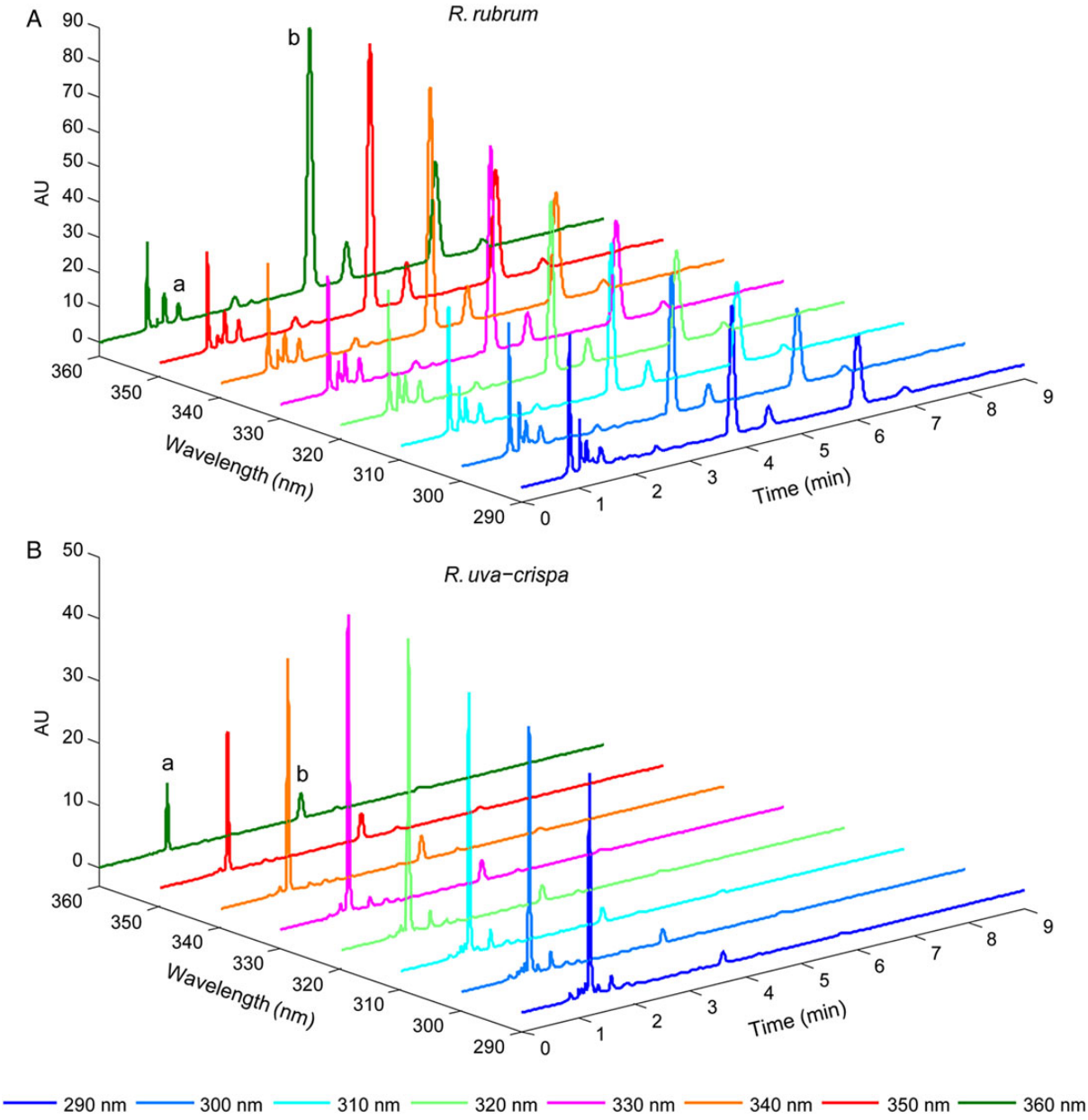


Figure 4. Multi-wavelength UPLC chromatograms of CA (a) and RUT (b) in *R. rubrum* (A) and *R. uva-crispa* (B). This figure is available in black and white in print and in color at *JCS* online.

plant samples were dried at room the temperature and deposited in Ankara University Faculty of Pharmacy Herbarium (AEF), Turkey. Collection sites and times with collector names and herbarium numbers of the analyzed plant samples are: *R. rubrum*: B5 Kayseri, 07.viii.2008, *G. Kendir, A. Tek* (AEF 25898); *R. biebersteinii*: A8 Rize, 01.viii.2008, *G. Kendir, B. Berberoğlu, Ü. Kendir* (AEF 25900); *R. nigrum*: B7 Tunceli, 19.vi.2005, *M. Koyuncu, N. Arslan, A. Gümüşçü* (AEF 23792); *R. uva-crispa*: A4 Ankara, 09.v.2008, 13.vi.2008, 30.v.2008, 24.iv.2009, *G. Kendir, Ö. Mumcu Arısan* (AEF 25903, 25904, 25905, 25906), 22.vi.2009, *G. Kendir* (AEF 25908); *R. alpinum*: A8 Rize, 01.viii.2008, *G. Kendir, Ü. Kendir* (AEF 25911); *R. orientale*: B5 Aksaray, 05.viii.2008, *G. Kendir, I. Başköse* (AEF 25917); *R. multiflorum*: B3 Afyon, 19.viii.2009, *G. Kendir, B. Tuna* (AEF 25923); *R. anatolica*: B8 Van, 14.vi.2008, *M. M. Hürkul* (AEF 25189). In the case of sample preparation, 5 g of the powdered leaves were used for the extraction procedure with 100 mL of methanol during 3 days at the temperature, 60°C by moving maceration technique. The extracted sample was filtered. The filtrated sample was evaporated to dryness at 40°C using a rotary evaporator, and 1.0 mg of the extracted sample was weighted and dissolved in 2 mL of methanol. The resulting solution was filtrated and injected into UPLC system to be obtained the chromatograms of the related compounds in samples of *Ribes* species.

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Results

UPLC-single calibration method

In the case of the conventional UPLC-single calibration approach, the UPLC chromatograms of the calibration solutions of CA and RUT in the linear range of 2.5–40.0 μg/mL were recorded using a PDA detector at the seven different wavelengths from 290 to 360 nm with a step of 10 nm. Similar chromatographs for the related plant samples were obtained under the above optimum chromatographic conditions. As an example, the UPLC chromatograms of CA and RUT were displayed in Figure 1A.

As it can be seen from Figure 1A, our chromatographic conditions gave us a good chromatographic separation of CA and RUT in the calibration step. In the suggested chromatographic conditions, the retention times of the resolved UPLC peaks were observed as 1.22 min for CA and 3.69 min for RUT. In the following experiments, the multi-wavelength UPLC chromatograms of the seven *Ribes* species containing both compounds were plotted and illustrated in Figure 1B for *R. alpinum*, Figure 2A and B for *R. anatolica* and *R. biebersteinii*, Figure 3A and B for *R. multiflorum* and *R. nigrum*, Figure 4A and B for *R. rubrum* and *R. uva-crispa*, respectively. Eight individual (single) calibration curves for each compound were calculated by applying the least squares regression analysis to concentration and UPLC peak areas obtained from 290, 300, 310, 320, 330, 340, 350 and 360 nm, respectively. The results of the calibration curves obtained by least squares regression analysis were summarized in Table I for both CA and RUT. The content of CA and RUT in leaves of the seven species was determined using the mentioned UPLC-single calibration graphs.

Table I
Regression Equations and Their Statistical Parameters Obtained by the Applications of the Conventional UPLC-Single Calibrations

λ (nm)	Regression equation	*r*	LOD	LOQ
CA				
290	$A_1 = 7132.1C_{CA} + 6502.5$	0.9995	0.33	1.1
300	$A_2 = 7977.9C_{CA} + 5862.4$	0.9995	0.36	1.2
310	$A_3 = 8614.4C_{CA} + 4428.1$	0.9996	0.31	1.02
320	$A_4 = 9669.3C_{CA} + 7623.4$	0.9995	0.59	1.95
330	$A_5 = 9743.4C_{CA} + 5765.1$	0.9998	0.66	2.19
340	$A_6 = 7883.3C_{CA} + 2867.8$	0.999	0.39	1.3
350	$A_7 = 4303.3C_{CA} + 9494.1$	0.9996	0.56	1.87
360	$A_8 = 2358.2C_{CA} + 4583.5$	0.9996	0.5	1.67
RUT				
290	$A_1 = 2523.9\ C_{RUT} + 1284.5$	0.9995	0.69	2.28
300	$A_2 = 2787.9\ C_{RUT} - 568.4$	0.9999	0.68	2.25
310	$A_3 = 3005.1\ C_{RUT} - 870.2$	0.9998	0.62	2.07
320	$A_4 = 3193.6\ C_{RUT} + 2291.9$	0.9997	0.65	2.16
330	$A_5 = 4043.1\ C_{RUT} + 849.7$	0.9997	0.56	1.87
340	$A_6 = 4863.4\ C_{RUT} - 1659.7$	0.9988	0.58	1.94
350	$A_7 = 5407.7\ C_{RUT} - 165.1$	0.9996	0.51	1.7
360	$A_8 = 5309.6\ C_{RUT} - 1132.3$	0.9997	0.72	2.4

r, Regression coefficient; LOD, limit of detection (μg/mL); LOQ, limit of quantitation (μg/mL); *C*, concentration (μg/mL); *A*, UPLC peak area.

UPLC-multivariate calibration

For UPLC-multivariate approaches, PCR and PLS calibration models were considered for the simultaneous quantitative prediction of CA and RUT in plant samples in order to eliminate the chromatographic instrumentation noise, baseline error and many chromatographic column fluctuations. As described in conventional UPLC-single calibration method, the multi-wavelength UPLC chromatograms of the calibration set (*y*-block) consisting of nine random samples of CA and RUT in the linear range 2.5–40.0 μg/mL were recorded and indicated in Figure 1A for the concentration set 5 from calibration set samples. The multi-wavelength UPLC peak area set (*x*-block) was obtained by integration of the recorded UPLC chromatograms at the eight-wavelength set from 290 to 360 nm with the interval of 10 nm. The linear relationship of calibration set (*y*-block) and UPLC

Table II
Analysis Results of CA in Leaves of the *Ribes* Species Using the Conventional UPLC, UPLC-PCR and UPLC-PLS Methods

		CA (μg/mg)									
		Conventional UPLC method								UPLC-PCR	UPLC-PLS
		290 nm	300 nm	310 nm	320 nm	330 nm	340 nm	350 nm	360 nm	290–360 nm	
R. alpinum	Mean	7.59	7.55	7.58	7.48	7.72	7.23	7.35	7.51	7.59	7.54
	SD	0.21	0.41	0.17	0.09	0.19	0.19	0.32	0.44	0.13	0.11
	RSD	2.83	5.38	2.19	1.24	2.41	2.59	4.35	5.83	1.75	1.47
R. anatolica	Mean	4.68	4.5	4.67	4.46	4.63	4.63	4.58	4.56	4.63	4.61
	SD	0.13	0.09	0.14	0.11	0.14	0.11	0.11	0.14	0.08	0.07
	RSD	2.68	2.08	3.00	2.51	2.96	2.39	2.32	3.09	1.68	1.51
R. biebersteinii	Mean	10.63	10.5	10.73	10.63	10.78	10.58	10.68	10.47	10.68	10.67
	SD	0.22	0.3	0.23	0.27	0.22	0.28	0.23	0.29	0.10	0.10
	RSD	2.11	2.84	2.14	2.51	2.02	2.68	2.18	2.77	0.94	0.95
R. multiflorum	Mean	1.68	1.67	1.65	1.65	1.54	1.6	1.7	1.68	1.67	1.68
	SD	0.04	0.03	0.04	0.04	0.03	0.03	0.04	0.04	0.02	0.01
	RSD	2.23	2.08	2.64	2.65	1.79	1.82	2.47	2.09	1.00	0.78
R. nigrum	Mean	2.79	2.77	2.78	2.76	2.75	2.74	2.73	2.76	2.78	2.78
	SD	0.08	0.06	0.06	0.06	0.08	0.07	0.08	0.06	0.03	0.03
	RSD	2.96	2.17	2.22	2.28	2.91	2.64	2.89	2.18	1.12	0.93
R. rubrum	Mean	13.5	13.74	13.13	13.35	13.36	13.15	13.38	13.02	13.23	13.2
	SD	0.35	0.39	0.42	0.39	0.45	0.36	0.42	0.40	0.32	0.29
	RSD	2.61	2.84	3.16	2.96	3.34	2.70	3.14	3.06	2.42	2.20
R. uva-crispa	Mean	23.12	22.99	22.34	23.41	23.01	22.91	22.29	22.2	22.91	22.94
	SD	0.71	0.70	0.45	0.45	0.45	0.46	0.73	0.51	0.14	0.21
	RSD	3.08	3.03	2.01	1.94	1.96	2.00	3.29	2.31	0.59	0.92

SD, Standard deviation; RSD, relative standard deviation.

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Table III

Analysis Results of RUT in Leaves of the *Ribes* Species Using the Conventional UPLC, UPLC-PCR and UPLC-PLS Methods

		RUT (μg/mg)									
		Conventional UPLC method								UPLC-PCR	UPLC-PLS
		290 nm	300 nm	310 nm	320 nm	330 nm	340 nm	350 nm	360 nm	290–360 nm	
R. alpinum	Mean	1.86	1.86	1.87	1.81	1.91	1.83	1.84	1.82	1.86	1.86
	SD	0.05	0.04	0.04	0.05	0.05	0.05	0.04	0.06	0.03	0.02
	RSD	2.84	1.91	1.99	2.80	2.57	2.74	2.33	3.16	1.35	1.33
R. anatolica	Mean	19.88	20.41	20.42	19.38	19.47	20.63	19.4	20	19.84	19.92
	SD	0.63	0.39	0.36	0.59	0.44	0.34	0.33	0.39	0.19	0.18
	RSD	3.15	1.93	1.78	3.03	2.25	1.65	1.72	1.97	0.94	0.89
R. biebersteinii	Mean	28.93	30.01	30.03	29.02	29.26	29.78	28.55	29.2	29.18	29.25
	SD	0.93	0.89	1.18	0.91	1.15	1.11	0.83	1.15	0.86	0.79
	RSD	3.22	2.95	3.91	3.14	3.93	3.74	2.90	3.95	2.94	2.70
R. multiflorum	Mean	16.95	16.53	16.69	15.48	16.42	16.39	16.17	16.09	16.26	16.28
	SD	0.49	0.50	0.51	0.66	0.72	0.35	0.51	0.40	0.21	0.21
	RSD	2.90	3.00	3.05	4.28	4.36	2.16	3.14	2.47	1.27	1.26
R. nigrum	Mean	13.92	14.84	14.78	13.81	14	14.66	13.79	14.18	14.15	14.21
	SD	0.47	0.59	0.30	0.44	0.54	0.32	0.46	0.34	0.27	0.27
	RSD	3.35	3.99	2.03	3.17	3.83	2.16	3.35	2.38	1.92	1.90
R. rubrum	Mean	178.27	180.61	181.04	190.22	180.95	177.88	174.27	175.01	175.76	175.75
	SD	3.51	5.26	3.51	3.50	3.88	3.80	3.53	3.56	1.80	1.81
	RSD	1.97	2.91	1.94	1.84	2.15	2.13	2.03	2.03	1.02	1.03
R. uva-crispa	Mean	8.38	8.63	8.67	8.30	8.28	8.48	8.24	8.50	8.40	8.41
	SD	0.19	0.17	0.26	0.17	0.26	0.09	0.22	0.18	0.03	0.03
	RSD	2.25	2.02	2.97	2.03	3.17	1.03	2.68	2.07	0.39	0.35

SD, Standard deviation; RSD, relative standard deviation.

Table IV

Recovery Data of CA in its Artificial Samples Containing the Plant Extracts Using the Conventional UPLC, UPLC-PCR and UPLC-PLS Methods

		Conventional UPLC								UPLC-PCR	UPLC-PLS
		290 nm	300 nm	310 nm	320 nm	330 nm	340 nm	350 nm	360 nm	290–360 nm	
R. alpinum	Mean	105.3	103.7	104	104.1	103.4	98.7	106.9	107.6	103.6	102.7
	RSD	3.39	4.08	3.93	4.07	3.16	10.14	5.37	1.33	1.90	1.77
R. anatolica	Mean	97.7	104.2	104.8	104.2	99.2	97.4	108.4	105.8	101.6	101
	RSD	3.31	3.93	3.06	3.67	5.64	4.10	4.22	2.90	2.67	2.02
R. biebersteinii	Mean	101.2	103	98.5	104.3	98.8	99	107.5	107.1	101.9	101.7
	RSD	3.03	4.32	8.89	3.11	5.96	6.71	3.05	3.10	2.83	1.69
R. multiflorum	Mean	102	98.9	98	96.8	99.0	97.2	107.8	107	102.2	101.9
	RSD	2.93	8.43	5.49	3.38	5.75	3.29	4.33	3.10	2.27	2.23
R. nigrum	Mean	104.8	104.6	101.6	103.7	103.4	102.2	106.5	106.2	102.5	101.9
	RSD	3.79	4.58	2.44	3.54	3.08	3.84	3.17	4.53	1.90	1.75
R. rubrum	Mean	97.4	100.3	98.8	100.8	99.1	98.5	109.8	107.1	101.4	100.9
	RSD	3.78	7.55	6.24	6.02	5.66	6.76	5.06	4.44	2.59	2.64
R. uva-crispa	Mean	105.4	104.7	100.8	104.6	105	102.7	107.1	106.3	102.5	102.2
	RSD	3.51	4.58	2.25	3.03	5.96	3.77	5.06	3.62	1.68	1.17

Assay results were obtained from the average of nine different experiments ($n = 9$).

Table V

Recovery Data of RUT in its Artificial Samples Containing the Plant Extracts Using the Conventional UPLC, UPLC-PCR and UPLC-PLS Methods

		Conventional UPLC								UPLC-PCR	UPLC-PLS
		290 nm	300 nm	310 nm	320 nm	330 nm	340 nm	350 nm	360 nm	290–360 nm	
R. alpinum	Mean	103.9	98	98.4	104.6	101.2	97.6	99.2	97.8	99.7	99.6
	RSD	3.67	2.44	3.01	2.63	1.55	4.08	3.07	3.30	1.34	1.17
R. anatolica	Mean	104	98.2	98.5	104.7	101.3	97.7	99.3	98	99.4	99.5
	RSD	3.71	2.34	3.07	2.67	1.53	4.06	2.97	3.19	1.60	1.37
R. biebersteinii	Mean	103.9	98	98.4	104.6	101.2	97.6	99.2	97.8	99.5	99.6
	RSD	3.67	2.44	3.01	2.63	2.48	4.08	3.07	3.30	1.55	1.54
R. multiflorum	Mean	103.9	98	98.4	104.6	103.2	97.6	99.2	97.8	99.4	99.5
	RSD	3.67	2.44	3.01	2.63	2.79	4.08	3.07	3.30	1.64	1.28
R. nigrum	Mean	103.2	101.1	107.2	106.9	102.0	98.1	96.4	102.6	99.1	98.9
	RSD	3.57	2.07	3.62	3.16	3.80	2.59	3.24	6.57	1.48	1.39
R. rubrum	Mean	101.9	102.6	105.7	105	99.1	96.9	96.5	105.9	99.3	99.1
	RSD	2.23	2.22	3.53	3.00	5.66	3.09	3.54	4.47	1.40	1.45
R. uva-crispa	Mean	103.4	99.7	104.9	104.9	99.9	98	99.2	97.8	99.4	99.3
	RSD	3.55	1.35	3.86	3.86	2.43	4.02	3.07	3.30	1.64	1.54

Assay results were obtained from the average of nine different experiments ($n = 9$).

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Table VI
Analysis Results of CA in Intra- and Inter-day Samples by Conventional UPLC, UPLC-PCR and UPLC-PLS Approaches

CA, Intra-day ($n = 3$)

Concentration (μg/mL)		Conventional UPLC approach												UPLC-PCR		UPLC-PLS	
		290 nm		300 nm		310 nm		320 nm		330 nm		340 nm		350 nm		360 nm		290–360 nm		290–360 nm	
RUT	CA	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %
10	10	9.57	2.63	9.58	3.77	9.68	3.74	10.17	3.45	9.73	3.14	9.87	4.22	9.83	3.27	9.93	3.24	9.93	1.54	9.97	1.16
25	25	25.40	2.73	25.73	3.16	24.80	3.04	25.03	2.89	24.80	2.91	24.27	2.65	24.43	2.25	23.93	2.52	24.92	1.68	25.00	1.60
35	35	35.50	2.82	36.00	2.78	35.67	2.88	35.53	2.13	35.13	2.02	34.20	2.11	35.07	3.14	35.53	2.83	35.17	1.15	34.90	1.04

CA, Inter-day ($n = 3$)

Concentration (μg/mL)		Conventional UPLC approach												UPLC-PCR		UPLC-PLS	
		290 nm		300 nm		310 nm		320 nm		330 nm		340 nm		350 nm		360 nm		290–360 nm		290–360 nm	
RUT	CA	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %
10	10	9.57	2.63	9.67	2.15	9.53	2.64	10.30	2.57	9.67	2.60	9.72	2.08	9.57	2.63	9.67	2.39	10.07	1.52	10.05	1.32
25	25	24.80	2.91	24.93	2.67	25.33	2.28	25.83	2.96	24.67	2.34	25.20	2.86	24.30	2.88	24.17	2.39	25.10	1.44	24.93	1.23
35	35	35.23	2.48	35.57	2.29	35.67	2.14	35.63	2.18	34.73	2.72	35.47	2.96	35.77	2.44	35.13	2.81	35.10	1.14	35.02	0.97

peak area set (x-block) for CA and RUT were used for the construction of the UPLC-multivariate calibration models, respectively. In practice, the UPLC-PCR and UPLC-PLS calibration models were built by applying the PCR and PLS algorithms to the x- and y-blocks, respectively. The ability function of the proposed UPLC-PCR and UPLC-PLS calibration models were evaluated by plotting and calculating the actual measurements versus predicted values in the calibration and prediction steps.

The simultaneous prediction of the amounts of CA and RUT in leaves samples of *Ribes* species were performed using the constructed UPLC-PCR and PCR-PLS calibration models.

Analysis of plant samples

The developed and validated conventional UPLC, UPLC-PCR and UPLC-PLS approaches were applied for the simultaneous quantitative estimation of the contents of CA and RUT in the extracted samples consisting of leaves of seven different *Ribes* species collected in different locations of Turkey. The multi-wavelength UPLC chromatograms obtained by application of the UPLC instrumentation to the plant samples were shown in Figure 1B for *R. alpinum*, Figure 2A and B for *R. anatolica* and *R. bieberstеinii*, Figure 3A and B for *R. multiflorum* and *R. nigrum* and Figure 4A and B for *R. rubrum* and *R. uva-crispa*. By using the proposed UPLC-single, UPLC-PCR, UPLC-PLS calibrations, the analysis results corresponding to the contents of CA and RUT in seven different *Ribes* species were summarized in Tables II and III, respectively. A good coincidence was reported for the assay results. In Tables II and III, the analysis results with standard deviations (SDs) and relative standard deviations (RSDs) were obtained from average of the five replicates. As can be seen in these tables, UPLC-multivariate calibrations (UPLC-PCR and UPLC-PLS) gave lower SDs and RSDs than conventional UPLC calibration approaches. According to numerical values of SDs and RSDs in the assay results of CA and RUT in the plant samples, it may be considered that UPLC-PCR and UPLC-PLS provided higher accuracy than conventional UPLC method. This indicates that the improved UPLC-PCR and UPLC-PLS could be eliminated the effects of random experimental and instrumentation errors.

Discussion

Applicability and validity of the improved UPLC-single and -multivariate approaches

In the application of the conventional UPLC method based on UPLC-single calibrations, a good linearity was observed for the eight regression graphs for each of CA and RUT in the working range of 2.5–40 μg/mL. The linearity of the regression equations for CA and RUT were evaluated using the regression coefficients, as shown in Table I. The limit of quantitation (LOD = $3 \times S/m$) and limit of detection (LOQ = $10 \times S/m$) were calculated using standard deviation (S) of intercept of the linear regression curve. The values of LOQ, LOD, regression coefficients (r), intercept (n) and slope (m) of regression equations with their standard errors were presented in Table I for both compounds. The predictive ability of the UPLC-PCR and UPLC-PLS calibration models were tested by using the difference between the actual and predicted concentrations in the calibration and prediction steps. In order to select the optimal factor number, cross validation procedure was used to obtain a highest predictive ability of

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Table VII
Analysis Results of RUT in Intra- and Inter-day Samples by Conventional UPLC, UPLC-PCR and UPLC-PLS Approaches

RUT, Intra-day ($n = 3$)

Concentration (μg/mL)		Conventional UPLC approach												UPLC-PCR		UPLC-PLS	
		290 nm		300 nm		310 nm		320 nm		330 nm		340 nm		350 nm		360 nm		290–360 nm		290–360 nm	
RUT	CA	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %
10	10	9.63	3.17	9.50	2.79	9.55	2.92	10.10	2.97	9.63	3.00	9.83	3.27	9.73	3.61	9.87	3.84	9.90	2.02	9.93	1.54
25	25	25.20	2.86	25.63	2.82	24.93	3.04	25.13	3.59	25.17	3.03	24.83	3.08	24.30	3.27	24.17	3.16	25.10	2.21	24.97	2.21
35	35	35.50	2.73	35.73	3.97	36.10	3.19	35.83	3.56	34.37	3.19	34.40	3.02	35.07	3.14	35.17	3.04	34.93	1.58	35.10	1.87

RUT, Inter-day ($n = 3$)

Concentration (μg/mL)		Conventional UPLC approach												UPLC-PCR		UPLC-PLS	
		290 nm		300 nm		310 nm		320 nm		330 nm		340 nm		350 nm		360 nm		290–360 nm		290–360 nm	
RUT	CA	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %	Found (μg/mL)	RSD %
10	10	9.60	3.61	9.73	3.30	9.57	3.19	10.13	3.01	9.73	2.97	9.78	3.08	9.63	2.16	9.93	3.24	10.10	1.98	9.97	1.53
25	25	24.87	3.25	25.30	2.77	25.67	2.98	25.93	3.48	25.20	3.64	25.13	2.82	24.20	3.23	24.60	2.67	24.97	1.85	24.90	1.45
35	35	35.13	2.87	35.73	3.08	35.83	2.90	35.90	3.42	34.80	3.04	35.57	3.38	35.93	3.07	35.30	3.22	34.80	2.01	35.40	1.49

the proposed models. After this procedure, first one factor and first two factors for CA and RUT were found to be optimum factors for the applicability of UPLC-PCR and UPLC-PLS.

For the evaluation of the accuracy and precision of the conventional UPLC, UPLC-PCR and UPLC-PLS methods, the recovery studies for nine different experiments were performed by the analysis of spiking plant samples with standard solutions of CA and RUT. For the preparation of spiking plant samples, the standard solutions of CA and RUT in nine different concentration levels from 2.5 and 40.0 μg/mL were added to each plant sample solutions corresponding to *R. alpinum*, *R. anatolica, R. biebersteinii*, *R. multiflorum, R. nigrum*, *R. rubrum* and *R. uva-crispa*. In the preparation of spiking plant samples, one of them was prepared without adding the standard solutions of pure compounds (CA and RUT) to the real sample solution of the analyzed plant. This was used for quantify the amounts of CA and RUT in the related plant sample. After subtracting the plant's quantities of CA and RUT from their total amounts in spiking plant samples, obtained by application of the proposed conventional UPLC and chemometric UPLC approaches, the recoveries results were calculated for both compounds. Recoveries, standard deviations and relative standard deviations for CA and RUT were depicted in Tables IV and V, respectively. In addition, the repeatability (intra-day precision) and for intermediate (inter-day) precision studies were carried out by using three different concentration levels (low:10 μg/mL, medium:25 μg/mL and high:35 μg/mL). These inter- and intra-day experiments were repeated three times for each compound. The results of the inter- and intra-day assays for CA and RUT were indicated in Tables VI and VII, respectively. Can be seen from these tables, the precision and accuracy of the analysis results provided by UPLC-PCR and UPLC-PLS calibration models were found to be better than that of the conventional UPLC approach. This proved that the use of the chemometric UPLC approaches for the analysis of complex plant samples was more suitable than conventional UPLC method.

Conclusions

In this study, the newly developed conventional UPLC-single, UPLC-PCR and UPLC-PLS calibration approaches were applied to simultaneous quantitative analysis of the content of CA and RUT in leaves of *Ribes* species and satisfactory assay results were obtained. In previous studies, the presence of CA and RUT in *Ribes nigrum* leaves have been reported (2, 3). It was concluded that the analysis results provided by the proposed UPLC-single and -multivariate methods developed gave us a good agreement with those obtained by the above studies. In addition, the UPLC-single and -multivariate calibrations proposed in this work provided us fast analysis (or short runtime) with higher peak resolution, accuracy, precise and low cost than that obtained the traditional HPLC method in previous research (9, 11, 14). When the UPLC method for the analysis of CA and RUT proposed in this study was compared with the literature methods, our method provides shorter runtime for the determination of the related substances (37–39). In comparison with the proposed UPLC-multivariate and conventional UPLC-single calibrations, UPLC-PCR and UPLC-PLS provided low relative standard deviation than that of the developed conventional UPLC for the quantitative estimation of the contents of CA and RUT in seven different *Ribes* species.

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