A validated UHPLC method for the determination of caffeoylquinic and di-caffeoylquinic acids in green coffee extracts using an RP-Amide fused-core column

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ABSTRACT

The presented work describes the development and validation of a rapid UHPLC-UV method using a fused core particle column with an RP-Amide stationary phase for the separation and quantitative analysis of caffeoylquinic and di-caffeoylquinic acids in green coffee extracts. Three caffeoylquinic acids (3-caffeoylquinic acid, 4-caffeoylquinic acid, and 5-caffeoylquinic acid) and two di-caffeoylquinic acids (1,3-di-caffeoylquinic acid, and 3,5-di-caffeoylquinic acid) were separated and analyzed in 8 min. That was possible due to the unique selectivity of the RP-Amide stationary phase for the analyzed acids. The retention behavior of all analytes under different compositions of the mobile phase on different columns was evaluated in this study. The optimal chromatographic separation was performed using an Ascentis Express RP-Amide (100 × 2.1 mm) fused-core column with a particle size of 2.7 µm at a temperature of 30 $^\circ$C. For validation of the newly developed method, acetonitrile was used as mobile phase B and 5% formic acid, filtrated through a 0.22 µm filter, was used as mobile phase A. They were delivered at a flow rate of 0.9 mL min^{-1} according to the elution gradient program. The detection wavelength was set at 325 nm. A solid-liquid extraction with a solution of methanol and a 5% water solution of formic acid (25 + 75 v/v) using an ultrasonic bath was chosen for the preparation of the available commercial samples of food supplements containing a green coffee extract. Recoveries for all analyzed acids were 98.2–101.0% and the relative standard deviation ranged from 0.3% to 1.4% for intra-day and from 0.3% to 3.0% for inter-day repeatability. The limits of detection were in the range of 0.30–0.53 g mL^{-1}.

1. Introduction

Isomers of chlorogenic acid (CGA) are phenolic compounds, cinnamic acid derivatives, with biological effects mostly related to their antioxidant and anti-inflammatory activities. Caffeoylquinic acids (CQA) and di-caffeoylquinic acids (di-CQA) are the main derivatives of chlorogenic acid, which is found in plant extracts [1]. Today, green coffee extract is being used to produce food supplements, because it is a major source of chlorogenic acid [2,3]. These food supplements have attracted a lot of attention for their promise of quick weight loss. Among the health benefits attributed to CGA derivatives are a reduced relative risk of cardiovascular disease and type 2 diabetes mellitus [4,5], antibacterial activity [6], anti-inflammatory effects [7], and the ability to slow the release of

glucose into the bloodstream after consuming a meal [8,9]. Nevertheless, the content of caffeoylquinic acids in green coffee extracts might differ depending on the extraction processes used, the loss of compounds during the manufacturing process and the source of the green coffee bean raw materials.

In this presented study, a new UHPLC method for the separation and determination of three caffeoylquinic acids (3-CQA, 4-CQA, and 5-CQA) and two di-caffeoylquinic acids (1,3-di-CQA, and 3,5-di-CQA), which were found in samples of food supplements containing green coffee extract, was developed. Three caffeoylquinic acids are also known as chlorogenic acid (3-CQA), cryptochlorogenic acid (4-CQA) and neochlorogenic acid (5-CQA). Isochlorogenic acid A is another name for 3,5-di-caffeoylquinic acid. Despite the great popularity of green coffee beans and food supplements containing green coffee extract, there are many general problems associated with food supplements (e.g. a legal classification, a lack of guarantees of efficiency and safety, the purity and stability of used substances, undeclared substances, etc.), as well as with the

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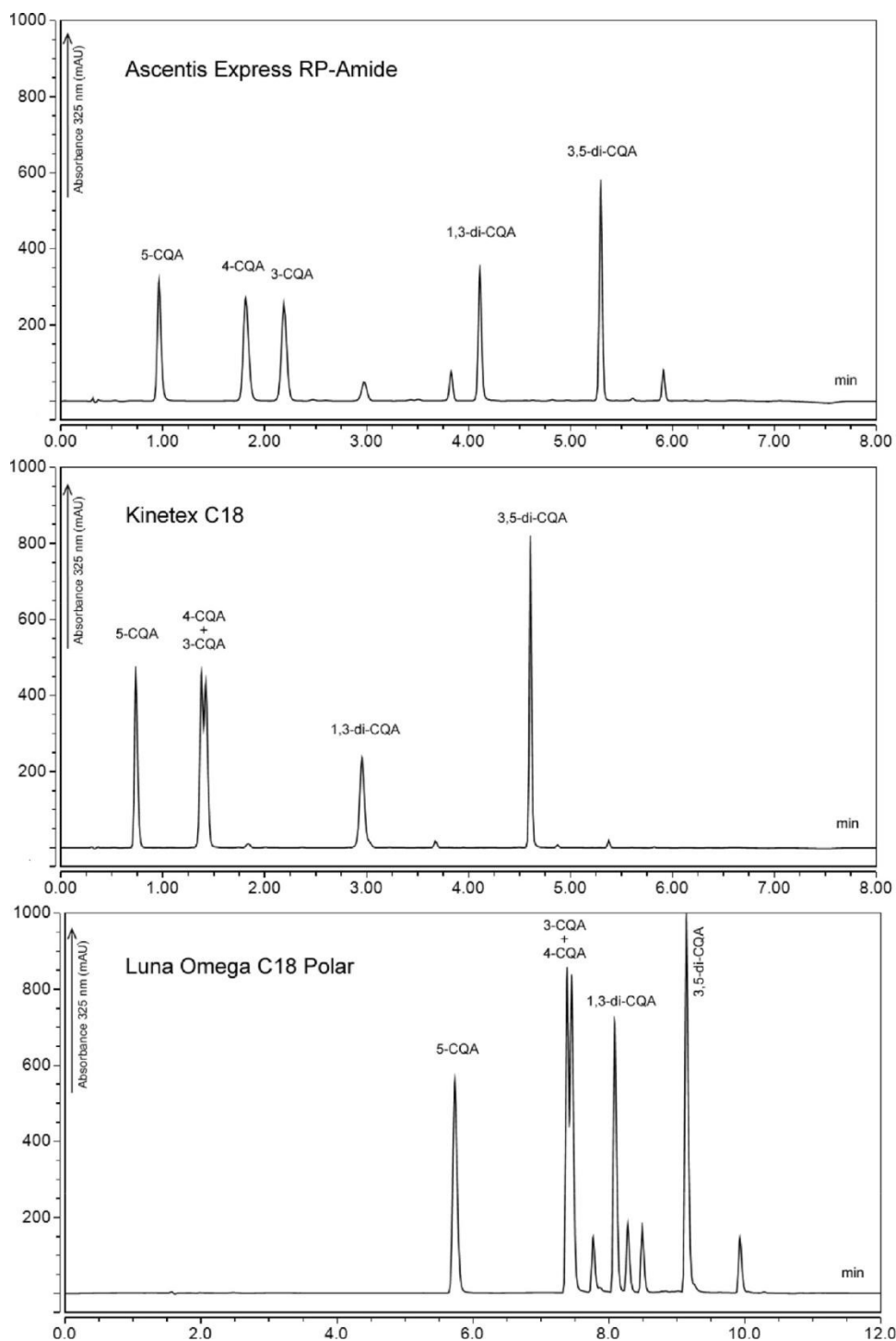E-mail address: satinsky@faf.cuni.cz (D. Šatínský).

Fig. 1. Comparison of caffeoylquinic acids separation on three different stationary phases: Ascentis Express RP-Amide (100 × 2.1 mm, 2.7 µm), Kinetex C18 (100 × 2.1 mm, 2.6 µm), and Luna Omega C18 Polar (150 × 2.1 mm, 1.6 µm) using the optimal chromatographic conditions and 2% formic acid as mobile phase A for all columns.

plant extracts themselves. Producers declare the content of a plant extract at a certain amount in their preparation, but this information tells us absolutely nothing about the content of biologically active substances (chlorogenic acid and its derivatives in this case). As can be seen in previous studies, the potencies of plant extracts vary in different food supplement preparations [10,11]. There is a huge contradiction between the amount of used extract and the content of active substances in one dose, which can have a substantial effect on dosing and lead to an overdose by consumers or to an inefficacy of preparation. To maintain the consumers' trust in

the safety and efficacy of products containing plant extracts, new modern analytical methods and studies for the quality assurance of food supplement preparations must be developed.

Several analytical methods for the determination of caffeoylquinic acids in plant materials [12–15], roasted coffee [16,17], brewed coffee, human plasma, urine extracts [1,18], and dietary supplements [19,20] have been published. Liquid chromatography coupled with PDA and MS detection is the most commonly used method for the determination of caffeoylquinic acids [12–20]. Even though mass spectrometry detection provides for the struc-
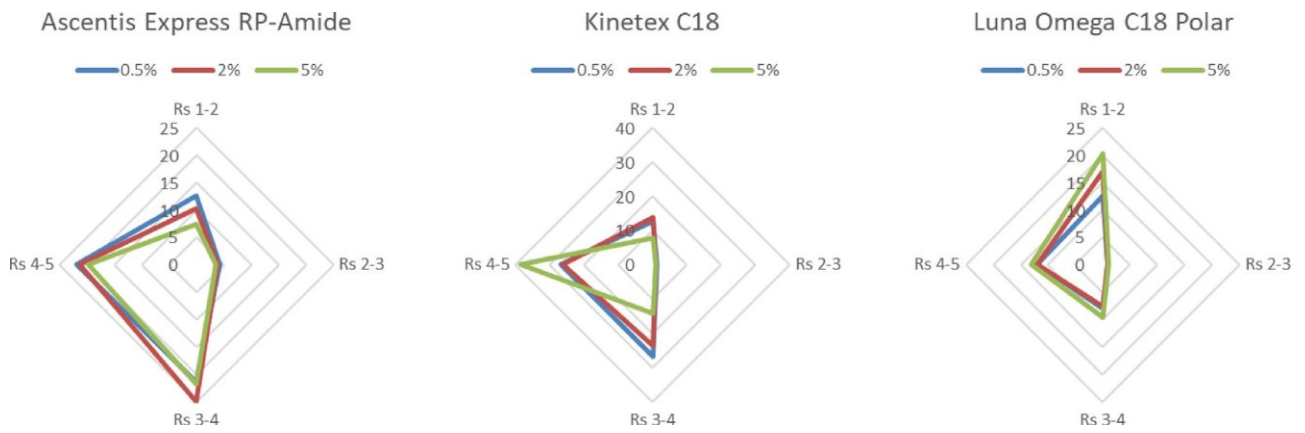
Fig. 2. Comparison of peaks resolutions of all analyzed acids on three different stationary phases: Ascentis Express RP-Amide (100 × 2.1 mm, 2.7 μm), Kinetex C18 (100 × 2.1 mm, 2.6 μm), and Luna Omega C18 Polar (150 × 2.1 mm, 1.6 μm) using the optimal chromatographic conditions for each column and different strengths of mobile phase A (0.5; 2; and 5% water solution of formic acid).
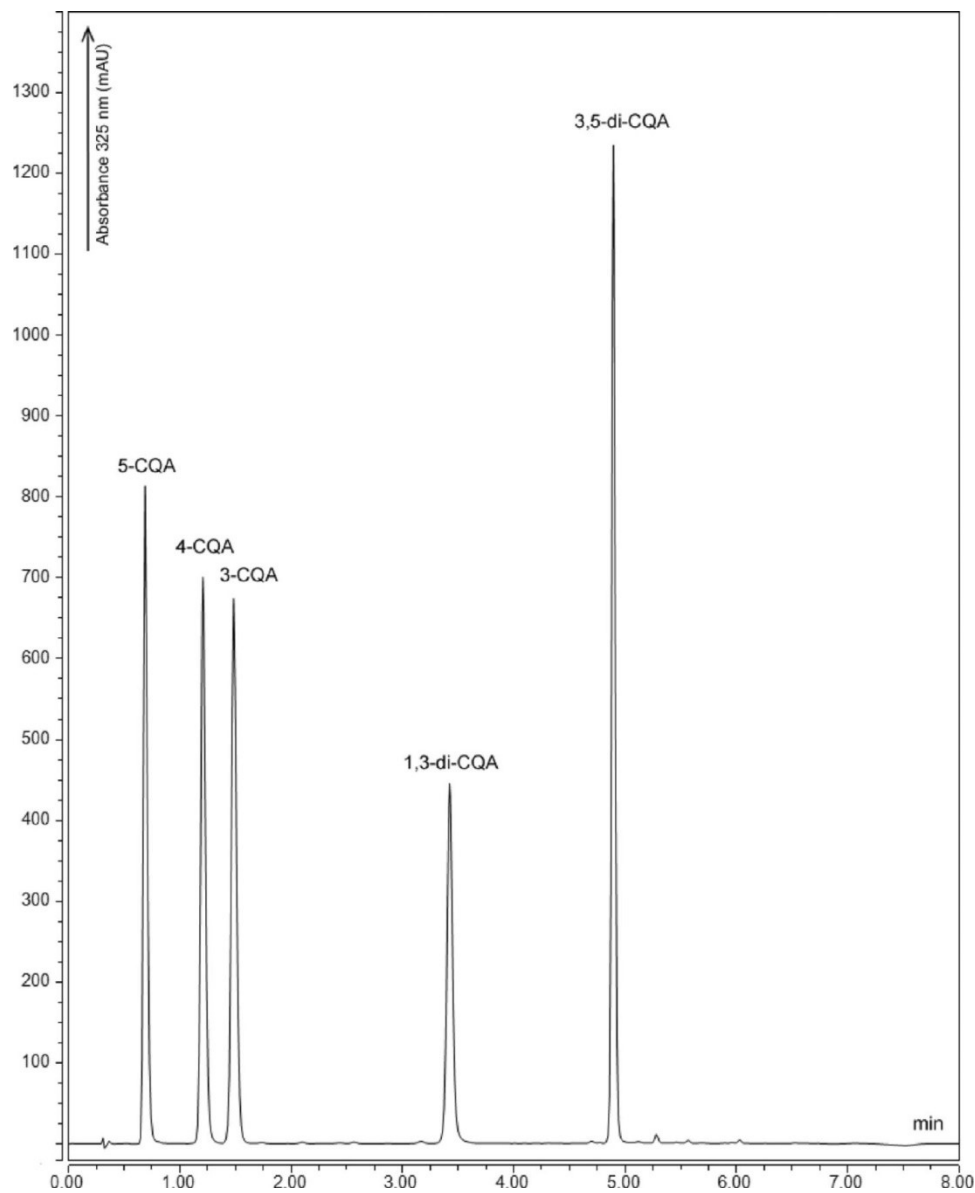
Fig. 3. Chromatogram of three caffeoylquinic acids (3-CQA, 4-CQA, and 5-CQA) and two di-caffeoylquinic acids (1,3-di-CQA, and 3,5-di-CQA) separation in standard solution performed on the Ascentis Express RP-Amide (100 × 2.1 mm) fused-core column with a particle size of 2.7 μm at 30 °C. Acetonitrile and 5% formic acid used as components of mobile phase were delivered at a flow rate of 0.9 mL min^{-1} according to the elution gradient program. The detection wavelength was set at 325 nmgr3.

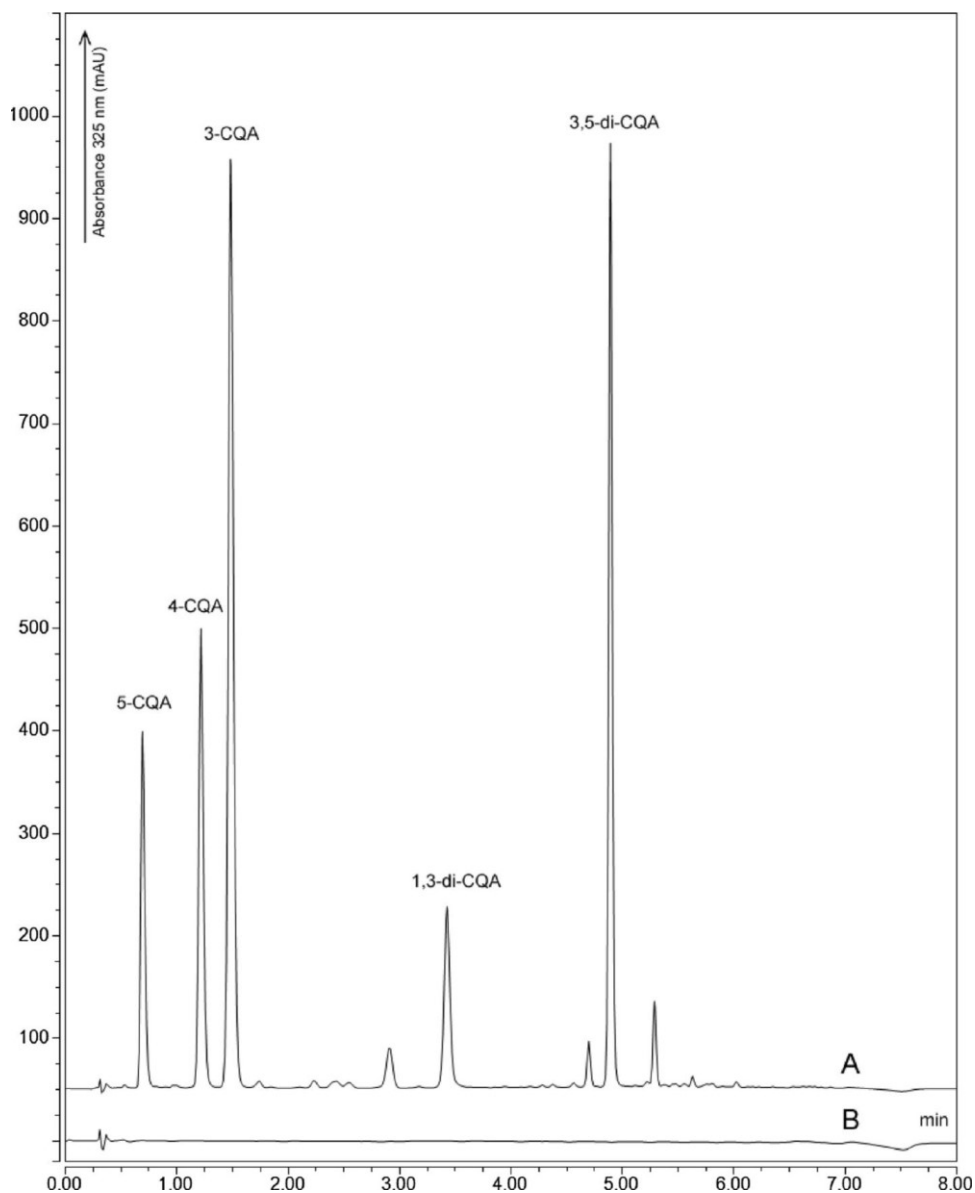**Fig. 4.** Chromatogram of sample (capsules with green coffee extract) spiked with all analyzed caffeoylquinic and di-caffeoylquinic acids (A), and blank injection (B) - overlaid.

tural characterization and identification of different caffeoylquinic acids, the MS-based quantitative multi-component analysis in plant materials is associated with unpredictable matrix effects and a limited number of expensive isotope-labelled internal standards. Therefore, a photo-diode-array detector is still widely used for the validation of analytical methods for the determination of chlorogenic acid derivatives in different samples, mostly at the absorption maximum of all compounds around 325 nm [12–16,20,21]. One limitation of PDA-based methods for analyzing compounds with similar spectral properties is the potential for the miss-identification of analytes, especially in the absence of authentic standards. Columns with only a conventional C18 stationary phase have been selected for the separation of caffeoylquinic acids [12–15,19,20] and di-caffeoylquinic acids [16,21,22] in different matrices. When performing routine sample analyses, many reported chromatographic methods are unsuitable due to their long runtimes, which usually last more than 40 min.

The aim of this study was to develop and validate a new chromatographic method for the fast and precise determination of

caffeoylquinic and di-caffeoylquinic acids that were found in seven food supplement samples containing green coffee extract. As a secondary objective, a comparison of the selectivity and separation efficiency of several stationary phases (RP-Amide, Polar C18, C18 PS, C18, CN, Phenyl-hexyl, and PFP) was carried out to verify that the RP-Amide stationary phase is the most suitable for the separation of caffeoylquinic and di-caffeoylquinic acids in respect of the resolution and symmetry of all peaks. The total number of peaks separated in different samples was higher than just the five caffeoylquinic and di- caffeoylquinic acids which were quantified due to the presence of other CQA derivatives in the samples in minor amounts. Finally, a retention study of all analytes under different compositions of the mobile phase was evaluated for the RP-Amide stationary phase to present that this phase has a different selectivity for polar acids than other tested stationary phases, especially C18 phases with fully porous and core-shell particles which are exclusively used to analyze caffeoylquinic acids, but are not at all suitable for the fast separation of these analytes.

Table 1
System suitability test results.

Analyte	t_R [a]	Repeatability		S [b]	R_S [c]	P_C [d]
		t_R, RSD (%)	Peak area, RSD (%)			
5-CQA	0.68	0.19	0.20	1.29	–	40.47
4-CQA	1.20	0.12	0.21	1.26	7.34	35.09
3-CQA	1.46	0.21	0.31	1.12	3.40	31.61
1,3-di-CQA	3.38	0.04	0.22	1.10	21.47	27.79
3,5-di-CQA	4.87	0.03	0.24	1.07	19.71	46.45

[a] Retention time, min.
[b] Symmetry factor.
[c] Resolution of peaks.
[d] Peak capacity.

Table 2
The analytical characteristic of the developed method (standard calibrations, regression coefficients and standard errors, detection and quantification limits).

Analyte	Calibration range ($\mu g \, mL^{-1}$)	Slope	Intercept	Regression standard error	R^2	LOD ($\mu g \, mL^{-1}$)	LOQ ($\mu g \, mL^{-1}$)
3-CQA	1–1000	0.1140 ± 0.0007	-1.1018 ± 0.2980	0.8546	0.9996	0.33	1.10
4-CQA	1–450	0.1153 ± 0.0015	-0.0342 ± 0.4213	0.8253	0.9987	0.35	1.15
5-CQA	1–450	0.1165 ± 0.0015	0.0046 ± 0.3931	0.7700	0.9988	0.30	0.99
1,3-di-CQA	2–450	0.0942 ± 0.0013	-0.0394 ± 0.3334	0.6531	0.9986	0.53	1.78
3,5-di-CQA	1–450	0.1377 ± 0.0015	0.0367 ± 0.4113	0.9927	0.9988	0.22	0.73

Table 3
Accuracy (recovery) and precision of the UHPLC method.

Analyte	Recovery (%)		Intra-day precision (%)	Inter-day precision(%)
	Level 1 [a]	Level 2 [b]		
3-CQA	99.4	99.0	0.27	0.38
4-CQA	98.8	98.5	0.33	0.33
5-CQA	98.8	99.5	0.28	0.33
1,3-di-CQA	101.0	99.9	0.27	1.25
3,5-di-CQA	98.2	98.9	1.37	2.95

[a] Concentration 400 $\mu g \, mL^{-1}$ for 3-CQA; 150 $\mu g \, mL^{-1}$ for all the other compounds.
[b] Concentration 650 $\mu g \, mL^{-1}$ for 3-CQA; 300 $\mu g \, mL^{-1}$ for all the other compounds.

2. Materials and methods

2.1. Chemicals and reagents

The HPLC-grade solvent used was methanol, and the LC–MS grade was acetonitrile, both of which were supplied by Sigma-Aldrich. All reagents used were of analytical grade or better. Ultra-pure water was obtained using a Milli-Q system. Commercially available reference standard 3-caffeoylquinic acid (≥95%), 4-caffeoylquinic acid (≥98%), and 5-caffeoylquinic acid (≥98%) were purchased from Sigma-Aldrich. Reference standards of 1,3-di-caffeoylquinic acid (≥98%), and 3,5-di-caffeoylquinic acid (≥98%) were purchased from Extrasynthese.

2.2. Characteristics of the samples

For the determination of caffeoylquinic and di-caffeoylquinic acids under the validated chromatographic conditions, seven different food supplement samples containing green coffee extract (capsules with declared amounts of extracts ranging from 200 to 500 mg) were used. According to the labels on the food supplement containers, only two preparations contained pure extract with nothing else in the capsules. The rest of the preparations were multi-componential and contained other biologically active substances or some excipients (mostly fillers and lubricants).

Table 4
Robustness of the validated UHPLC method and stability of samples.

| Parameter		Analyte				
		3-CQA	4-CQA	5-CQA	1,3-di-CQA	3,5-di-CQA
Column temperature +5 °C (35 °C)	Peak area	+0.5%	+0.9%	+0.2%	−0.3%	+0.8%
	t_R	−9.4%	−9.4%	−6.4%	−10.5%	−3.2%
Column temperature −5 °C (25 °C)	Peak area	+0.5%	+0.9%	+0.1%	−0.8%	+0.1%
	t_R	+11.5%	+11.3%	+7.6%	+8.2%	+3.0%
Mobile phase A +0.5% HCOOH (4.5%)	Peak area	−0.2%	−0.3%	+0.1%	−0.7%	−0.9%
	t_R	+9.0%	+8.4%	+6.6%	+5.8%	+2.0%
Mobile phase A −0.5% HCOOH (5.5%)	Peak area	−0.6%	−1.6%	−0.6%	−1.9%	−1.7%
	t_R	−5.9%	−6.3%	−5.4%	−5.5%	−1.9%
Stability of samples in the autosampler for 3 days (20 °C)	Peak area	−3.0%	−1.6%	−3.1%	+0.2%	−2.5%
Stability of samples in the freezer for 14 days (-10°C)	Peak area	+0.0%	+0.2%	-0.2%	+0.0%	-0.3%
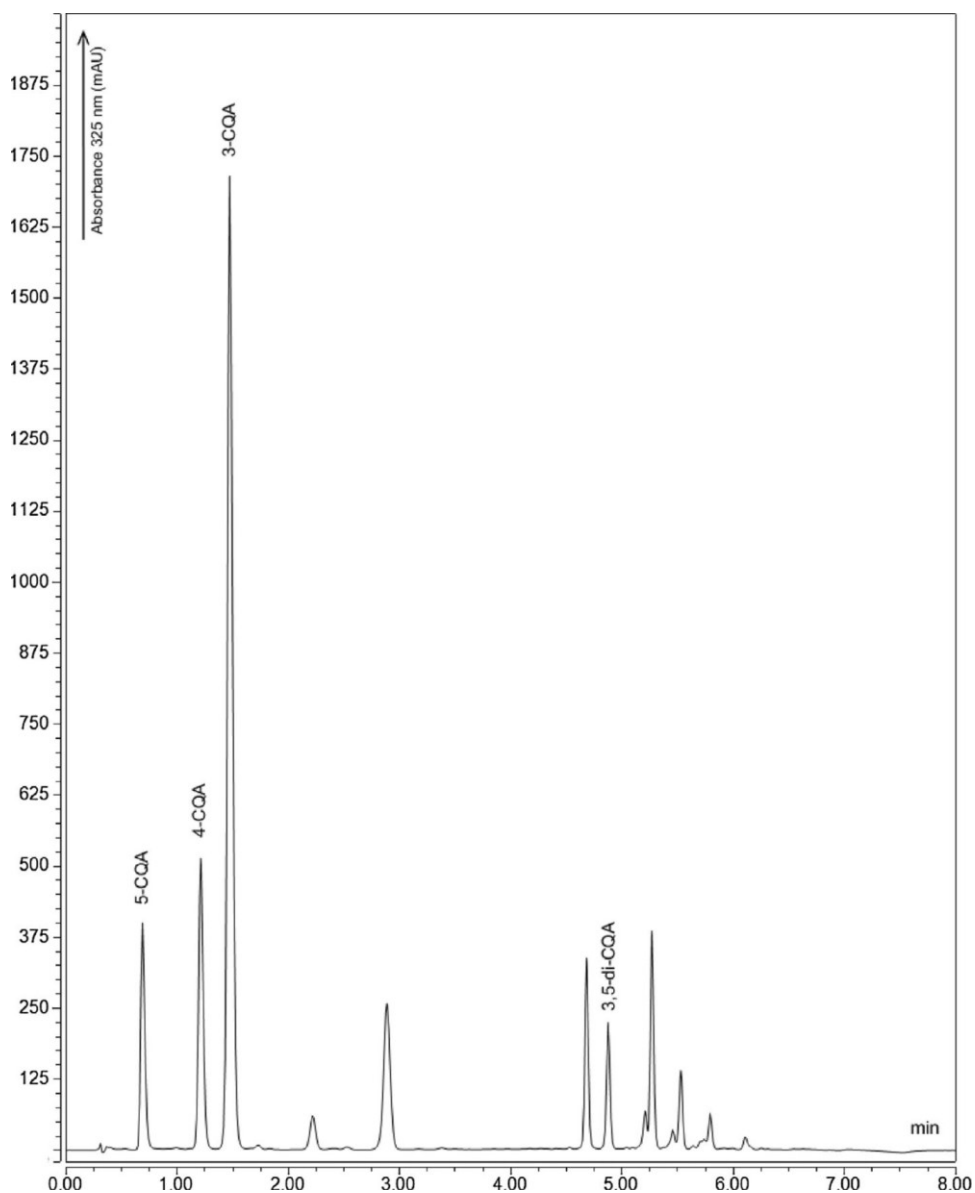
Fig. 5. Chromatogram of caffeoylquinic and di-caffeoylquinic acids determination in food supplement sample (Vito life – capsules with green coffee extract) obtained under validated conditions.

Table 5
Determination of caffeoylquinic and di-caffeoylquinic acids in various food supplement preparations with green coffee extract.

Food supplement with green coffee extract (brand name)	Found amount in one capsule (mg)						Found amount in 100 mg of preparation (mg)	Found amount in 100 mg of extract (mg)
	3-CQA	4-CQA	5-CQA	1,3-di-CQA	3,5-di-CQA	TOTAL	TOTAL	TOTAL
Zelena kava max	98.5 ± 0.3	20.3 ± 0.1	14.1 ± 0.0	<LOQ	12.0 ± 0.2	144.9	28.8	29.0
Vito life kyselina chlorogenova	69.0 ± 0.3	18.3 ± 0.1	13.0 ± 0.0	<LOQ	5.4 ± 0.3	105.7	26.3	26.4
Zelena kava bylinny extrakt	61.7 ± 0.1	9.7 ± 0.0	6.6 ± 0.0	<LOQ	4.8 ± 0.0	82.8	20.8	41.4
Zelena kava extra	16.5 ± 0.2	3.5 ± 0.0	2.3 ± 0.0	<LOQ	1.0 ± 0.1	23.3	5.8	11.7
Zelena kava premium	14.8 ± 0.0	2.5 ± 0.0	1.5 ± 0.0	<LOQ	0.6 ± 0.0	19.4	3.8	78.0
Vito life zelena kava	9.5 ± 0.1	2.2 ± 0.0	1.5 ± 0.0	<LOQ	0.6 ± 0.0	13.8	3.5	3.8
Kilostop	1.8 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	<LOQ	0.1 ± 0.0	2.6	0.4	40.2

2.3. Instrumentation and the chromatographic conditions

The UHPLC chromatographic system by Thermo Scientific, the Dionex UltiMate™ 3000 RSLC system equipped with a binary pump, an autosampler, a column oven, and a Diode Array detector was used. System control, data acquisition, and data evaluation were performed using the Dionex − Chromeleon™ 7.2 Chromatography Data System. Acetonitrile was used as mobile phase B and a 5% water solution of formic acid, filtrated through a 0.22 m filter, was used as mobile phase A. They were delivered at a flow rate of 0.9 mL min^{-1} according to the following elution gradient program: 0.0–2.5 min 7–10% mobile phase B, 2.5–6.0 min 10–33% mobile

phase B, 6.0–6.5 min 33–60% mobile phase B, 6.5–7.0 min 60-7% mobile phase B, and 7.0–8.0 min 7% mobile phase B. Separation was performed using an Ascentis Express RP-Amide (100 × 2.1 mm) fused-core column with a particle size of 2.7 μm at a temperature of 30 °C. A KrudKatcher Ultra filter was used to protect the column from microparticulates. The peaks of the separated caffeoylquinic acids and di-caffeoylquinic acids were detected at a wavelength of 325 nm. The PDA detector collected data at a sampling rate of 100 points per sec. The temperature of the autosampler cooling system was set at 20 °C to maintain the stability of the samples. An injection volume of 2 μL was used. A solution of methanol and water (50 + 50 v/v) was selected as the needle wash solvent.

2.4. Preparation of the standard solutions

A standard stock solution of 1000 μg mL^{-1} was prepared for the chlorogenic acid (3-CQA) reference standard and a standard stock solution of 450 μg mL^{-1} was individually prepared for each reference standard of the other two caffeoylquinic acids (4-CQA and 5-CQA) and both di-caffeoylquinic acids (1,3-di-CQA and 3,5-di-CQA) in a solution of methanol and a 5% water solution of formic acid (25 + 75 v/v). All the stock solutions were stored at −10 °C in a dark place until use. The mixed standard solution was prepared by mixing aliquots of each of the standard solutions and was used for the system suitability test.

2.5. Sample preparation

A mass of 0.2–0.6 g of a homogenized content of twenty capsules of a preparation containing green coffee extract was accurately weighed into a 50-mL volumetric flask. The mixture was extracted for 10 min with the help of an ultrasonic bath into 50 mL of methanol and a 5% water solution of formic acid solution (25 + 75 v/v). Subsequently, the solution was filtrated through a 0.22 μm PTFE filter into a glass vial.

2.6. Method validation

To evaluate the suitability of the whole procedure for the determination and quantification of five caffeoylquinic acids in food supplement samples, the developed method was validated in accordance with the ICH validation guideline [23]. For the method specificity estimate, spiked samples and blank solutions prepared from real samples in the same way were analyzed. The recovery and accuracy of the method were performed by spiking known concentrations of standard solutions at two different concentration levels. The limit of detection (LOD) and the limit of quantification (LOQ) were defined as the lowest concentration of analyzed compounds that gave a reproducible peak with threefold (3ϭ) and tenfold (10ϭ) variations, respectively, of the baseline noise obtained from the blank injection and signals of analytes.

3. Results and discussion

3.1. Optimization of the chromatographic separation conditions

Among the most commonly used chromatographic conditions for the determination of caffeoylquinic acids belong conventional HPLC systems with a C18 stationary phase, columns with length of 250 mm, gradient elution and long runtimes of analyses. In our study, the UHPLC system was chosen for method development and optimization to achieve a higher efficiency of peak separation within a short analysis time. To obtain the best chromatographic conditions, the stationary phase, mobile phase composition, column oven temperature and the flowrate of the mobile phase were optimized.

In the first step, several columns with different chemistry of stationary phases were tested: C18 with core-shell (Kinetex) and fully porous particles (Luna Omega Polar and PS), pentafluorophenyl, phenyl-hexyl (both core-shell Kinetex), nitrile (ES-CN), and RP-Amide (both core-shell Ascentis Express). The mobile phase consisting of a water solution of formic acid was chosen to suppress the ionization of the carboxyl group and the phenolic hydroxyls of caffeoylquinic acids. Acetonitrile was selected as the organic part of the mobile phase because it was more suitable for a faster elution. A resolution of chlorogenic acid (3-CQA) and cryptochlorogenic acid (4-CQA) was identified as being critical for the acceptable selectivity of the stationary phases under different isocratic and gradient elution (5–10% of acetonitrile in the mobile phase or the start of the gradient program at 5% of the organic part).

In the second step of method development, a retention study of the analyzed compounds on three columns (Ascentis Express RP-Amide 100 × 2.1 mm, 2.7 μm; Kinetex C18 100 × 2.1 mm, 2.6 μm; and Luna Omega C18 Polar 150 × 2.1 mm, 1.6 μm) with potentially desired selectivity was performed. The effect of the formic acid water solution strength in mobile phase A (0.5, 2.0, and 5.0%) on the resolution of five peaks of caffeoylquinic acids was examined using an optimized gradient program for each column at a column oven temperature of 30 °C. Three chromatograms for each column using 2% formic acid as component A of the mobile phase in Fig. 1 show how the selectivity of the RP-Amide stationary phase (core-shell column) differs from the other two types of C18 phase (with core-shell and fully porous particles). A comparison of all three columns regarding the resolution of all peaks using different compositions of mobile phase A visualized on radar charts is given in Fig. 2. With increasing concentration of formic acid in mobile phase A elution of all peaks was accelerated on RP-Amide column but as can be seen with preserved satisfactory resolution of all peaks. However, the retention behavior of the analytes on the other two columns was quite different. The effect of mobile phase A composition on the resolution of peak numbers 2 and 3 was almost insignificant on all three tested columns.

To validate the analytical method, the Ascentis Express RP-Amide column and gradient elution consisting of acetonitrile and 5% formic acid were selected because it led to the best resolution of all peaks in the shortest runtime. The most appropriate flow-rate of the mobile phase was found to be 0.9 mL min^{-1} with respect to the system backpressure and column oven temperature. The effect of temperature on the separation was investigated and the results are shown in Table 4 as a part of the method's robustness. A column oven temperature of 30 °C was found to be optimal. Based on the UV–vis spectra and absorption maxima of all analyzed acids, a detection wavelength at 325 nm was selected for monitoring and performing a quantitative analysis. The optimal chromatographic conditions, as a separation shown in Fig. 3 for the standard solution and in Fig. 4 for the spiked real sample, are reliable for the determination and quantification of all caffeoylquinic and di-caffeoylquinic acids concerning resolution, peak capacity, peak symmetry, and short analysis time.

3.2. Optimization of extraction conditions

To optimize the extraction of caffeoylquinic and di-caffeoylquinic acids from green coffee extracts in food supplement samples, several aspects affecting extraction were studied. A water solution of methanol (40–80%) is the most commonly used extraction solvent in papers dealing with analyses of caffeoylquinic acids in different matrices [1,14,16,19]. A method based on ultrasound-assisted solid-liquid extraction was selected as the most suitable for further optimization. Experiments with the composition of the extraction solvent, regarding the content of the organic part and formic acid, as well as the sonification time, were carried

out to achieve the best yields of extracted caffeoylquinic and di-caffeoylquinic acids. During method optimization, it was discovered that all determined caffeoylquinic and di-caffeoylquinic acids were extracted from food supplement samples with the highest yields into the mixtures of methanol and the 5% water solution of formic acid in the range of (50 + 50 v/v) to (25 + 75 v/v) within the shortest sonification times.

The best chromatographic separation and symmetry of peaks, especially those which were eluted in the first two minutes of the gradient, were achieved with the mixture of methanol and the 5% water solution of formic acid (25 + 75 v/v). That allowed using this extraction solvent without any further dilution of the sample solutions. The optimal time of extraction was found to be 10 min of sonification. Longer sonification than 10 min had no effect on the extraction solutions, however extraction times longer than 20 min led to heating of the extraction solvent and decreased yields of analytes, probably due to the thermal instability of the analytes in aqueous solutions. The results from the analysis showed that the use of 0.2–0.6 g of each sample of food supplement containing green coffee extract was sufficient for extracting and precisely quantifying all caffeoylquinic and di-caffeoylquinic acids. All the final solutions were filtrated through a 0.22 μm filter and immediately placed into an autosampler before being injected into the UHPLC system.

3.3. Validation

Validation of the method included an evaluation of the following performance parameters: calibration, the repeatability of injections, precision, accuracy (recovery), detection limits, quantification limits, selectivity, robustness, and the stability of the samples in order to evaluate the reliability of the results provided by the proposed method.

3.4. System suitability test

Within the method validation, the parameters of the UHPLC system suitability were measured and evaluated from six replicate injections of the mixed standard solution (Section 2.4) before the real samples were analyzed. The system suitability test for the developed method established the following parameters: the repeatability of the retention times (t_R) and peak areas, the symme-try factor (S), the resolution of the peaks (R_S), and peak capacity (P_C). All parameters maintained RSD values lower than 1.0% (Table 1). All tested parameters met the acceptance criteria during the analyses. A representative chromatogram of three caffeoylquinic acids (3-CQA, 4-CQA, and 5-CQA) and two di-caffeoylquinic acids (1,3-di-CQA, and 3,5-di-CQA) separation in a standard solution is provided in Fig. 3.

3.5. Calibration

To test the detector response, standard calibration solutions at six concentration levels were prepared. The linearity of the proposed method was studied in the range of 1–1000 μg mL^{-1} for chlorogenic acid (3-CQA) and in the range of 1–450 μg mL^{-1} for all other analyzed compounds. The difference in the upper limit of quantification between chlorogenic acid and the other analytes was due to the significantly higher content of chlorogenic acid in food supplement preparations than the content of other caffeoylquinic and di-caffeoylquinic acids. According to the values of the regression parameters for the calibration curves, all the compounds showed a good correlation ($R^2 \geq 0.999$). The slopes, intercepts, regression coefficients, regression standard errors, and calibration ranges are presented in Table 2.

3.6. Accuracy, precision, and selectivity

The precision of the developed method was determined by analyzing independently prepared real samples of one food supplement preparation spiked with a 1,3-di-CQA solution (this acid was found in all preparations under the limit of quantification). Intra-day precision was measured on six repeated preparations in one day. Inter-day precision was established over a period of three days. The accuracy was expressed in terms of compound recovery at two different concentration levels (400 and 650 μg mL^{-1} for chlorogenic acid − 3-CQA; 150 and 300 μg mL^{-1} for the rest of the analyzed compounds). The data summarized in Table 3 indicates that the method showed satisfactory recoveries for all analyzed compounds and were between 98.2 and 101.0%. The relative standard deviation ranged from 0.3% to 1.4% for intra-day and from 0.3% to 3.0% for inter-day precision for all analytes. These results demonstrate that the precision and accuracy of the newly-developed method are suitable for performing routine sample analyses.

All the analyzed caffeoylquinic and di-caffeoylquinic acids were successfully separated within 5 min and all the peaks were free of interferences at the retention time of the analytes. For selectivity, no interferences were detected in the corresponding retention times of the target compounds by comparing the chromatograms of the spiked sample and blank injection (Fig. 4). The results showed that the developed method has high selectivity for measuring caffeoylquinic and di-caffeoylquinic acids in plant extracts.

3.7. LOD and LOQ

The detection and quantification limits of the analyzed compounds were determined as the lowest concentrations that could be determined to be statistically different from a blank injection and give a signal-to-noise ratio of 3 (S/N = 3) and 10 (S/N = 10), respectively. The results of LOD and LOQ for the five analytes in the spiked solution are listed in Table 2. The limits of detection ranged from 0.30 to 0.53 μg mL^{-1}. These results demonstrate that the method is sufficiently sensitive.

3.8. Robustness

The robustness of the newly-developed method was demonstrated by measuring the effect of small and deliberate changes made to the analytical parameters on retention time and peak area counts of all analyzed compounds. The parameters that were taken into consideration were column oven temperature (±5 °C) and composition of the mobile phase A (±0.5% of the water solution of formic acid). The results of all tested conditions are given in Table 4. While the retention times of the peaks were affected by changes made to column temperature and the composition of mobile phase A, the peak areas of all analyzed caffeoylquinic and di-caffeoylquinic acids remained unaffected. The minimal variations of the peak areas of all compounds in samples measured under altered conditions confirm that the validated method is robust enough and minor changes in the analytical parameters will not affect the precise determination of analyzed compounds.

3.9. Stability

The stability of the standard stock solutions when stored at −10 °C for 2 weeks in a freezer and the stability of samples main-tained at 20 °C in an autosampler for 72 h were investigated due to the suspicion of thermal instability in aqueous solutions of all analyzed compounds based on observations made during method development and in the published data [24]. As shown in Table 4, the stock solutions were found to be stable for periods longer than 2 weeks at −10 °C and sample solutions in the autosampler

for 72 h at 20 °C. The minimal variations of the peak areas in the stored solutions show that the optimized and validated conditions of the method are robust enough and a longer storage of vials in an autosampler will not significantly affect the results of the analyses.

3.10. Determination of caffeoylquinic and di-caffeoylquinic acids in food supplements

The newly-developed method was applied to determine caffeoylquinic and di-caffeoylquinic acids in food supplements. The samples were commercially available on the local market. Its sufficient accuracy, high precision and very low limits of quantification showed that the method is reliable to quantitatively analyze all the caffeoylquinic and di-caffeoylquinic acids in real commercial preparations. Each peak found in the samples was identified by comparing its retention time to that of the respective reference standard. The confirmation of identity of each compound found in plant extract samples was performed with HRMS system Synapt G2Si. HRMS and MS/MS data are presented in Supplementary materials. A representative chromatogram of food supplement sample analysis ('Vito life' – capsules with green coffee extract) is shown in Fig. 5. The results of caffeoylquinic and di-caffeoylquinic acids content in different food supplements, including the total amount of analyzed compounds in single-dose form (hard capsules), in 100 mg of preparation, and in 100 mg of extract used in each food supplement (the declared amount of extract in single-dose form was defined by the producers) are presented in Table 5. In all analyzed food supplement preparations, chlorogenic acid (3-CQA) was found in the highest amount followed by cryptochlorogenic acid (4-CQA) and neochlorogenic acid (5-CQA). 3,5-di-caffeoylquinic acid was determined as a minor component in green coffee extracts. 1,3-di-caffeoylquinic acid was not present in any sample above its limit of quantification. As can be seen, the content of all analyzed compounds varied in different samples. However, a huge contradiction between the amount of used extract and the content of caffeoylquinic and di-caffeoylquinic acids in one capsule of each preparation was found. The highest potency of green coffee extract was seen in the 'Zelena kava premium' preparation, which had 78.0 mg of caffeoylquinic and di-caffeoylquinic acids in 100 mg of extract. Nevertheless, the total amount of caffeoylquinic and di-caffeoylquinic acids in one dose was relatively low due to the lower amount of used green coffee extract in one capsule.

4. Conclusion

A fast method for the separation and simultaneous quantification of five caffeoylquinic and di-caffeoylquinic acids found in green coffee extracts in food supplement capsules was established. A solid-liquid extraction method using an ultrasonic bath for just 10 min and an extraction solvent of methanol and a 5% water solution of formic acid (25 + 75 v/v) was the fastest and most efficient sample pre-treatment procedure used for all compound extractions from food supplement preparations. A fused-core chromatographic column with an RP-Amide stationary phase provided the optimal separation of all determined compounds in a short time and in the presence of other compounds found in real samples giving a response at a detection wavelength of 325 nm. The RP-Amide phase offered a type of selectivity that enabled the separation of all compounds at the desired resolution and symmetry of all peaks. A gradient elution program consisting of acetonitrile and 5% formic acid at a column temperature of 30 °C was used in the validated method. Due to the fast sample preparation procedure and the short analysis time of 8 min, the newly developed method is reliable for performing routine analyses of green coffee extracts used in food supplement preparations.

In conclusion, the obtained results showed a great variety of caffeoylquinic and di-caffeoylquinic acids content in the tested food supplements. Chlorogenic acid (3-CQA) was found to be a major component in all analyzed extracts in the tested food supplement samples. Moreover, a retention study of all analytes under different mobile phase compositions was evaluated for the RP-Amide stationary phase to show that this stationary phase has a different selectivity for polar acids than other tested stationary phases. C18 stationary phases with fully porous or core-shell particles are exclusively used to analyze caffeoylquinic and di-caffeoylquinic acids, but are not at all suitable for the very fast separation of these analytes. Only the RP-Amide stationary phase in combination with a 5% water solution of formic acid as mobile phase A and acetonitrile as mobile phase B enabled the fast separation of all analytes with a satisfactory resolution, especially for chlorogenic and cryptochlorogenic acids, which was not achieved with any other stationary phase in an acceptable runtime. A contribution of our newly developed method could be highlighted by comparison with previous validated method used for analysis of plant extracts. A method used by Craig et al. [25] is capable to resolve chlorogenic and cryptochlorogenic acids in 5 min, whereas our method in less than 1.6 min. In addition, the total runtime of our method is 3-times shorter and sensitivity for all compounds is approximately 10-times higher.

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