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A new kinetic method using UV-VIS spectrophotometry for determination of caffeic acid in propolis

Ein neues kinetisches Verfahren zur Bestimmung von Kaffeesäure in Propolis mittels UV-VIS-Spektrophotometrie

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Summary

The aim of this work is to develop an application kinetic-spectrophotometric procedure for the determination of caffeic acid (CA) in propolis. The method is based on oxidation reaction of CA by hydrogen peroxide in the presence of Cu (II) ions in alkaline solution. The reaction was monitored spectrophotometrically by measuring the rate of change of absorbance at 345 nm. The optimum operating conditions for reagent concentrations and temperature were established. Linear calibration curve was obtained in the range of 1.94 to 19.4 µg/ml with standard deviation from 2.77 to 4.15 %. The optimized conditions yielded a theoretical detection limit of 0.6 µg/ml based on 3.3So criterion. The developed method is sensitive, accurate and reproducible and could be used for routine analysis of CA in propolis.

Keywords: caffeic acid, interference, kinetic procedure, propolis

Zusammenfassung

Ziel dieser Arbeit ist die Entwicklung eines kinetisch-spektrophotometrischen Verfahrens zur Bestimmung von Kaffeesäure (KS) in Propolis. Die Methode basiert auf der Oxidationsreaktion von KS mit Wasserstoffperoxid unter Anwesenheit von Cu (II)-Ionen in alkalischer Lösung. Die Reaktion wurde spektrophotometrisch überprüft, indem die Änderungsrate der Absorption bei 345 nm gemessen wurde. Die optimalen Betriebsbedingungen hinsichtlich der Konzentration der Reagenzien sowie der Temperatur wurden ermittelt. Die lineare Kalibrierungskurve lag im Bereich von 1,94 to 19,4 µg/ml, mit einer Standardabweichung von 2,77 bis 4,15%. Die optimierten Bedingungen ergaben eine theoretische Nachweisgrenze von 0,6 µg/ml, basierend auf dem 3.3So-Kriterium. Die entwickelte Methode ist sensitiv, präzise sowie reproduzierbar und könnte bei der Routineanalyse von KS in Propolis zum Einsatz kommen.

Schlüsselwörter: Kaffeesäure, Interferenz, kinetisches Verfahren, Propolis

Journal of Food Safety and Food Quality 70, Heft 4 (2019), Seiten 91–124

Introduction

Caffeic acid (CA), 3,4-dihydroxycinnamic acid, is one of the most abundant hydroxycinnamic acids found naturally in fruits, vegetables, cereals, legumes, coffee, and tea. It is also found in wine for human consumption as simple esters with quinic acid (Iglesias et al., 2009; Jiang et al., 2005).

CA and related compounds are well known radical scavengers and antioxidants in coffee beans with health-promoting attributes (Celik and Erdogan, 2008; Kanimozhi and Prasad, 2015). In addition, CA, multifunctional natural available organic acid plays a significant role in binding metal ions from the natural environment. The compound has two complexing sites in competition: the catechol group (dihydroxybenzene) and the carboxylic function.

Several coworkers reported the complexation of the compound with different metal ions in aqueous solutions: Al (III) (Cornard and Lapouge, 2006; Cornard et al., 2006), copper (II), Ni (II), Zn (II), Co (II) and iron (III) (Hynes and O'Coinceanainn, 2004). There are also reports on the complexation of caffeic acid with polyphenol and aromatic compounds investigated by spectroscopic and computational methods for advanced design and controllable carriers of drugs and food components (Górnas et al., 2009).

Propolis or "bee glue" is the generic name of the resinous product which is collected by bees from various plant sources (Burdock, 1998), and its composition varies with the source. Generally, it is composed of 50 % resin and vegetable balsam, 30 % wax, 10 % essential oils and aromatics, 5% pollen, and 5% other substances (Siheri et al., 2017). Propolis is well known from ancient times because of its biological and pharmacological properties, like immunomodulatory, antitumoral, antimicrobial, anti-inflammatory, and antioxidant effects (Alvarez-Suarez, 2017). Methods of the used extraction may influence its activity. The most used method is solid-liquid extraction (ethanol in different concentrations, methanol or water). The biological activity of propolis is mainly due to the presence of flavonoids, especially caffeic acid (CA) and its phenethyl ester (CAPE) (Santos-Buelga and González-Paramás, 2017).

Up to now, our colleagues (Tosic et al., 2017) were investigated the content of minerals in propolis and found that propolis from Serbia is rich in minerals. Since propolis is rich in phenolic acids, and particularly caffeic acid, we decided to investigate the content of caffeic acid in propolis by the application of new and simple UV/VIS method.

There are published UV-Vis spectrophotometric methods for the determination of CA, but they are not easy to perform and are not sufficiently sensitive, accurate and selective. The aim of the present work is the development of a simple, sensitive, specific, spectrophotometric method for the detection of CA in propolis, which does not need sophisticated instruments or special skills.

Materials and Methods

Apparatus

UV/Vis spectrophotometer (Perkin-Elmer Lambda 15) equipped with kinetic accessory and a temperature controlled cell was used.

HPLC analysis was carried out using the HPLC system 1200 (Agilent) series with a semi-preparative diode array detector, and a Zorbax Eclipse XDB-C18 Semi-Prep, 5um, 9.4x250 mm column.

Reagents

Stock solution (0.1 mol/l) of NaOH (Merck, Darmstadt, Germany) was prepared in deionized water.

A stock solution ($1.0 \cdot 10^{-3}$ mol/l) of CA was prepared in absolute ethanol from CA powder. A stock solution of Cu (II) ($1.0 \cdot 10^{-3}$ mol/l) was prepared by dissolving $CuCl_2$ (J. T. Baker, USA) in water. Hydrogen peroxide solution (0.442 mol/l) was prepared from the 34 % reagent and stored at 4 °C.

For the preparation of all solutions, analytical reagent grade chemicals and deionized water (MicroMed High Purity Water System) were used. All used glassware was washed with aqueous HCl (1:1) and then with distilled water, and finally with deionized water.

General procedure

Prior to the first measurement, the instruments were run for 10 min to obtain good mechanical and thermal stability.

In the reaction mixture four compartments vessel, the solution of CA was placed in one compartment, NaOH in the second, in the third Cu (II), hydrogen peroxide and water (total volume: 10 ml) in the fourth compartment.

The vessel was thermostated at 25.0±0.1°C, and the reaction was initiated by vigorously shaking the reactants, which was followed by transferring the content to a cell, and the absorbance at 345 nm was measured using UV/VIS spectrophotometer every 30 s for 5–6 min against the blank prepared similarly. The rate of the reaction at different concentrations of each reactant was determined by measuring the slope of the linear part of the curves of the absorbance time plot.

Sample preparation

Samples of raw propolis were collected during 2017 by scraping the frames of bee hives that originated from three different regions of Serbia: Stara planina, Soko banja and Suva planina. The representative sample of a particular location was obtained through mixing the propolis from ten different apiaries. The samples were frozen and pulverized.

Five grams of propolis (in small pieces) was soaked into 10 ml of water, and then heated at 70 °C for 40 min in a thermostatic water bath system and filtered yielding the refined propolis, which was then placed in a constant temperature drying oven set at 80 °C until the constant weight. Thus obtained propolis samples were homogenized using an agate homogenizer and then stored in sealed glass vials until further analysis.

Methanol, ethanol and acetonitrile in different concentration were examined as extracting solvents for caffeic acid from propolis. The highest extraction yield was obtained with methanol 80 %.

In order to extract phenolic compounds, 3.50 g of the dried propolis samples was soaked into 10 ml of 80 % methanol for 24 h. The obtained solution was extracted using ultrasound-assisted procedure twice (each time for 20 min), and then evaporated using a rotary evaporator (at 65 °C) to dryness (Čižmárik and Matel, 1970).

The obtained sample was treated with 100 ml of the mobile phase, filtered through a filter (pore size 0.45 µm) and injected into the HPLC.

HPLC analysis

HPLC analysis was carried out using the HPLC system 1200 (Agilent) series with a diode array detector, and a Zorbax Eclipse XDB-C18 Semi-Prep, 5um, 9.4x250 mm column. Solvents used for separation were 0.1 % orthophosphoric acid in water (v/v) (eluent A) and 0.1% orthophosphoric acid in methanol (v/v) (eluent B). The gradient used was: 0–10 min, linear gradient from 40 % to 50 % B; 10–15 min, linear gradient from 50 % to 60 % B, maintain at 60 % B until 25 min. The flow rate was 1.0 ml/min. Detection wavelength was 330 nm. The sample injection volume was 10 µl. The chromatographic peaks of caffeic acid were confirmed by comparing their retention times and UV spectra with that of their reference standards. Working standard solutions were injected into the HPLC and peak area responses obtained. Standard graphs were prepared by plotting concentration versus area. Quantification was carried out from integrated peak areas of the samples using the corresponding standard graph (Wang et al., 2004).

Statistical analysis

The data were reported as the mean ± standard deviation *(SD)* for triplicates. The significance of inter-group differences was determined by the analysis of variance (ANOVA). The p-value < 0.05 was considered statistically significant (Statistical Analysis and Reporting System, ser Guide, version 1.0, 1MB, 1999).

Results

Kinetic studies

The kinetic data were processed using the differential variant of the tangent method (Svehla, 1993) due to the fact that a linear correlation exists between the absorbance at 345 nm and time during the first 6 min after mixing. To determine the lowest possible detectable concentration of CA, the conditions had to be optimized.

Effect of variables

Constant experimental parameters were kept while the dependence of the reaction rate on pH in NaOH solution (0.1 mol/l) in the range of 0.5–2.0·10^{-3} mol/l was studied. (Fig. 1).

The optimum value of the difference between the rates of non-substrate and substrate reactions was at a concentration of NaOH solution 1.0 ·10^{-3} mol/l and it was used for further work.

However, the correlation between the reaction rate and pH was not linear, so the logarithms of *tan*α were calculated and the gotten values were plotted *versus* pH. Using the obtained regression equations, the order of reaction was determined (0.8) in the NaOH concentration range (0.5·10^{-3}–2.0 ·10^{-3} mol/l).

The reaction rate dependence on the concentration of H_2O_2 was investigated in the range 0.442–8.84·10^{-2} mol/l. Oxidation reaction rate dependence of caffeic acid on hydroxide peroxide concentration was shown in Fig. 2 and it is an exponential function.

The correlation between the reaction rate and H_2O_2 concentration was not linear, so the logarithms of *tan*α were calculated and the gotten values were plotted *versus* the logarithms of H_2O_2 concentration. The order of reaction was minus 0.7 in the whole interval of H_2O_2 concentration. For further investigation, a concentration of H_2O_2 of 1.326·10^{-2} mol/l was selected.

How reaction rates depend on the concentrations of Cu (II) it was monitored over the range (1·10^{-6} mol/l–5·10^{-6} mol/l) (Fig. 3).

For further investigation, a concentration of Cu (II) of 2·10^{-6} mol/l was selected as the working value. Regarding Cu (II) concentration, the rate of substrate reaction was –1 order.

The optimal reaction conditions were:

$C_{NaOH} = 1 \cdot 10^{-2}$ *mol/l*, $C_{Cu(II)} = 2 \cdot 10^{-6}$ *mol/l*, $C_{H2O2} = 1.326 \cdot 10^{-2}$ *mol/l*,

$t = 25 \pm 0.1$ °C, $\lambda = 345$ *nm*

Taking into account the kinetics of the proposed indicator reaction, the kinetic equation (Eq. 1) for the reaction was derived (Ermer, 2001).

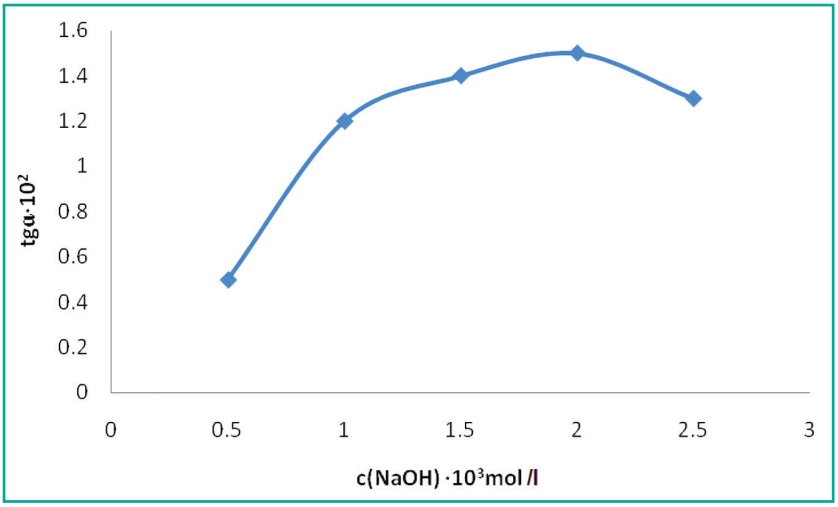

FIGURE 1: *Dependence of reaction rate on pH. Initial concentrations:* $C_{CA} = 8 \cdot 10^{-5}$ *mol/l,* $C_{Cu(II)} = 1 \cdot 10^{-6}$ *mol/l,* $C_{H2O2} = 8.825 \cdot 10^{-2}$ *mol/l,* $t = 25 \pm 0.1$ °C, $\lambda = 345$ *nm.*

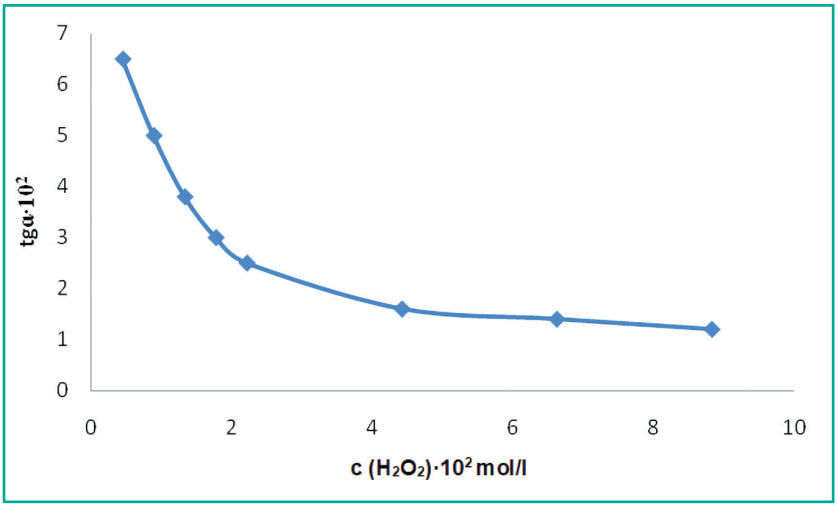

FIGURE 2: *Dependence of the reaction rate on* H_2O_2 *concentration. Initial concentrations:* $C_{CA} = 8 \cdot 10^{-5}$ *mol/l,* $C_{Cu(II)} = 1 \cdot 10^{-6}$ *mol/l,* $C_{NaOH} = 1 \cdot 10^{-2}$ *mol/l,* $t = 25 \pm 0.1$ °C, $\lambda = 345$ *nm.*

Journal of Food Safety and Food Quality 70, Heft 4 (2019), Seiten 91–124

$$\frac{dc}{dt} = k \cdot c^{0.8}_{NaOH} \cdot c^{-1}_{Cu} \cdot c^{-0.7}_{H_2O_2} \cdot c^{1}_{CA} \quad (1)$$

Concentrations of CA were varied (1.94–19.4 µg/ml) and a linear dependence was found between tg α and the concentration of CA (Fig. 4).

The kinetic equations (Eq. 2-4) for the reaction were derived on the basis of the kinetics of the indicator reaction proposed.

$$tg\alpha = 0.2387\, C_{CA} - 0.1214,\ R^2 = 0.9984,\ t{=}20{\pm}0.1\ °C \quad (2)$$
$$tg\alpha = 0.2625\, C_{CA} - 0.1168,\ R^2 = 0.9984,\ t{=}25{\pm}0.1\ °C \quad (3)$$
$$tg\alpha = 0.287\ C_{CA} - 0.0847,\ R^2 = 0.9982,\ t{=}30{\pm}0.1\ °C \quad (4)$$

where $tg\alpha$ corresponds to the slope of the linear part of the curve of the absorbance-time plot; C_{CA} is concentration of CA expressed as µg/ml and R is the coefficient of the correlation.

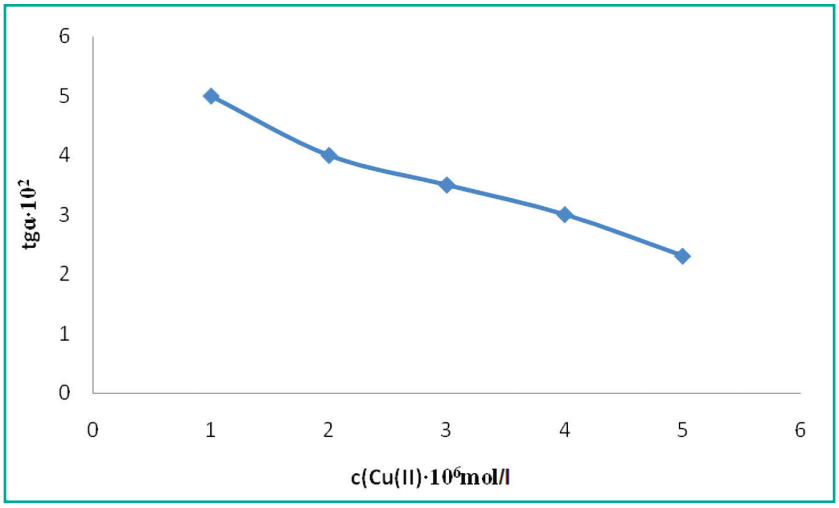

FIGURE 3: *Dependence of reaction rate on Cu (II) concentration. Initial concentrations: $C_{CA} = 8{\cdot}10^{-5}$ mol/l, $C_{NaOH} = 1{\cdot}10^{-2}$ mol/l, $C_{H2O2} = 1.326{\cdot}10^{-2}$ mol/l, t = 25 ± 0.1 °C, λ = 345 nm.*

Parameter value

The limit of detection (LOD) (Prichard and Bedson, 2001) was found according to Eq. 5,

$$c_L = \frac{3.3 \cdot S_0}{m} \quad (5)$$

where S_0 corresponds to the residual standard deviation of the calibration line, and m to the slope of the calibration line. LOD was found to be 0.6 µg/ml. The limit of quantification (LOQ) was found according to Eq. 6.

$$c_Q = \frac{10 \cdot S_0}{m} \quad (6)$$

It was found to be 1.8 µg/ml, which indicates that the method is sensitive.

The accuracy and the precision of the proposed method were investigated by performing the experiment five times at three different CA concentration levels (low, medium and high) (Table 1).

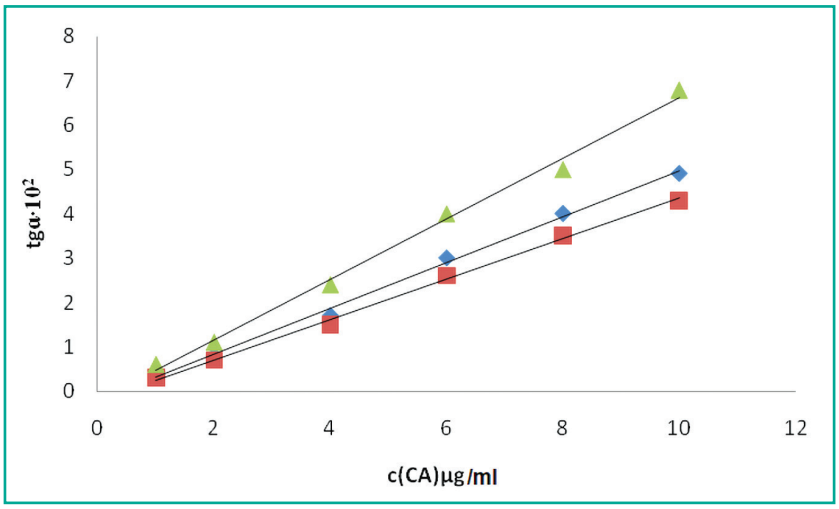

FIGURE 4: *Dependence of reaction rate on CA concentration. Initial concentrations: $C_{NaOH} = 1{\cdot}10^{-2}$ mol/l, $C_{Cu(II)} = 2{\cdot}10^{-6}$ mol/l, $C_{H2O2} = 1.326{\cdot}10^{-2}$ mol/l, t = 20±0.1 °C (red), t = 25±0.1 °C (blue), t = 30±0.1 °C (green), λ=345 nm.*

TABLE 1: *Accuracy and precision of the proposed method.*

Taken (µg/ml)	Found (µg/ml)	N	S	RSD %	$\frac{\bar{x}-\mu}{\mu} \cdot 100$
1.94	2.02	5	0.06	2.77	3.96
11.65	12.12	5	0.34	2.82	3.86
19.42	20.20	5	0.84	4.15	4.00

N: number of experiments from each sample

Interference studies

A systematic investigation of the possible interferences by species accompanying CA in natural pharmaceuticals was performed in order to find the selectivity of the method. For the established level of caffeic acid, 5 % variation of the average slope change measured (n=5) was fixed as the criterion of interference (Table 2).

Several ions and some substances-microcrystalline cellulose, starch, lactose, fructose, talc, magnesium stearate, were shown to have no interference. Contrary, severe interference was observed for ascorbic acid, citric acid, and other phenolic acids (in 1:1 ratio). The interference of caffeic acid phenethyl ester (CAPE) is not expected due to the extraction procedure of caffeic acid from propolis and polarity of the ester (Chen et al., 1996; Russo et al., 2002).

TABLE 2: *Tolerance ratio for foreign species in the determination of CA (11.65 µg/ml) under optimal conditions.*

Taken C_x/C_{CA}	Foreign species
100	Na^+, K^+, Ni^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, NO_3^-, CH_3COO^-, CO_3^{2-}, Cl^-
100	talc, fructose, lactose, starch, magnesium stearate, microcrystalline cellulose
10	Se^{4+}, Cd^{2+}, Mn^{2+}, Pb^{2+}, Cu^{2+}, Br^-, SO_4^{2-}
Interfere	ascorbic acid, glucose, citric acid, gallic acid, coumaric acid, ferulic acid

Applicability of the proposed method

A point hypothesis test was used to statistically compare our results with those of the standard HPLC method (Chen et al., 2012) (Table 3).

TABLE 3: *Determination of CA in propolis by the proposed method (kinetic) and standard HPLC methods.*

Sample	Content of CA HPLC method (µg/ml)	RSD (%)	Recovery (%)	Content of CA kinetic method (µg/ml)	F-test*	t-test**
Stara planina	3.05±0.20	1.86	99.20	3.00±0.15	1.83	1.26
Suva planina	2.70±0.30	2.11	98.76	2.60±0.20	1.73	1.13
Soko banja	2.60±0.25	1.49	99.54	2.50±0.20	2.06	1.36

*Data are based on the mean of five determinations; *Theoretical F-value (v1 = 4, v2 = 4) and t- value (v = 8) at 95 % confidence level are 6.39 and 2.306, respectively.

The accuracy was estimated by recovery tests performed for each analyte. The spiked propolis samples were prepared and analysed by the proposed HPLC-DAD method. The results are shown in Table 3. The average recoveries are in the range 98.76–99.54 % (RSD < 2.11 %); the results showed very good recoveries for the proposed analytical method.

Discussion

Using the differential tangential method, optimal conditions for the determination of the micro amounts of CA in the solution are determined.

$$C_{NaOH} = 1 \cdot 10^{-2}\ mol/l,\ C_{Cu(II)} = 2 \cdot 10^{-6}\ mol/l,$$
$$C_{H2O2} = 1.326 \cdot 10^{-2}\ mol/l\ ,\ t = 25 \pm 0.1\ °C,\ \lambda = 345\ nm$$

Under the experimental conditions, concentrations of CA were 1.94–19.4 µg/ml and a linear dependence was found between tg α and the concentration of CA. A linear calibration curve is constructed (1.94–19.4 µg/ml with the relative standard deviation of less than 4 %, and the detection limit of 0.6 µg/ml.

Examination of the selectivity of the method, *i.e.* the effects of numerous compounds and ions present in natural and pharmaceutical preparations, does not significantly influence the determination of CA (Tab. 3).

The developed method is accurate, sensitive, and reproducible and could be used for everyday analysis of CA. In comparison with other spectrophotometric methods, the method is more sensitive and selective. Propolis compounds have been analyzed by other different methods: thin-layer chromatography (TLC), gas chromatography-mass spectrometry (GC-MS) (Garcia Viguera et al., 1993), high performance liquid chromatography (HPLC) (Bankova et al., 1982), LC-MS and micellar electrokinetic capillary chromatography (MEKC) (Fontana et al., 2000).

The method was developed for the determination of CA in propolis and the results obtained are compared with those obtained by the HPLC method. Statistical analysis of the results (Tab. 3) showed that on the basis of calculated *F* and *t* values (95 % confidence levels) there are no significant differences between the performance of the proposed and the standard HPLC method. Therefore, the proposed spectrophotometric method could be used for the determination of CA in propolis. Comparing to HPLC methods the linearity intervals (LOD, LOQ) are in the range of previously published methods (Spagnol et al., 2016). Our method is suitable for the investigations and the control in areas without modern and expensive equipment and also for fieldwork.

The content of caffeic acid in propolis samples from different locations in the area of Southeast Serbia ranges from 2.5 to 3.0 %, which is in line with the results shown by the analysis of propolis from the territory of Croatia (continental part: 0.27–2.67 %, Adriatic part: 0–10.11 %) (Kosalec et al., 2003), Romania (1.15–1.54 %), Israel (1.23–1.51 %) (Croci et al., 2009), and Turkey (Anatolian part: 0.05–1.2 %, Kazan: 0.32 %, Marmaris: 18.54 %) (Kartal et al., 2002; Uzel et al., 2005). It is noteworthy that the samples from the areas close to the sea show higher concentrations of caffeic acid in the propolis samples.

Propolis is very useful due to its richness in minerals (Tosic et al., 2017) and natural phenolic compounds, especially caffeic acid that has very beneficial effects on health (Alvarez-Suarez, 2017).

Conclusions

The developed method is sensitive, accurate and reproducible and could be used for routine analysis of CA. No significant differences between the performance of the proposed and the standard HPLC method confirms the usefulness of using our method. Therefore, the proposed method could be used for the determination of CA in propolis.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Journal of Food Safety and Food Quality 70, Heft 4 (2019), Seiten 91–124

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