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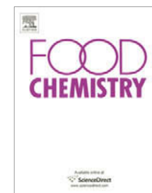

Analytical Methods

Multiresidue determination of tetracycline antibiotics in propolis by using HPLC-UV detection with ultrasonic-assisted extraction and two-step solid phase extraction

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An analytical method in propolis was developed and validated for the determination of four tetracyclines (TCs) by high performance liquid chromatography (HPLC) for the first time. After extraction by ultrasound, the extracting solution was subjected to Oasis HLB and weak cation-exchange cartridge to remove water-soluble and fat-soluble flavonoids, aromatic acids, terpenoid compounds, wax, and pollen debris. The calibration curves of fortified samples showed acceptable linear response ($R^2 > 0.99$) through a range of 100–5000 ng g^{-1} in 20 replicates of six concentrations and the analysis of variance (ANOVA) was performed to validate the regression data. The limit of quantification of four TCs were 100 and 150 ng g^{-1}, respectively. The recoveries of the four TCs for propolis samples spiked with 100–500 ng g^{-1} were in the range of 61.9–88.5% and the RSDs were between 4.80% and 13.2%. Traces of tetracycline were found in two out of 30 analysed real samples.

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1. Introduction

Propolis (bee glue) is a chemically complex resinous bee product that honeybees collect from leaf buds and exudates of plants of various plants. The honeybee uses it to strengthen the borders of combs as the building material; what is more, it is regarded as the "chemical weapon" of bees against pathogen microorganisms (Wollenweber, Hausen, & Greenaway, 1990). Propolis has been used extensively in folk medicine for many years because of the complex chemical compositions (Bankova, Castrom, & Marcucci, 2000), and there is evidence to suggest that propolis has several medicinal properties including antibacterial, antiviral, antitumor, anti-inflammatory, anticancer and immunomodulatory (Banskota et al., 2002; Bazo et al., 2002; Kimoto et al., 2001; Murad, Calvi, Soares, Bankova, & Sforcin, 2002; Sforcin, Fernandes, Lopes, Bankova, & Funari, 2000) and so on.

Tetracyclines (TCs) are broad-spectrum antibiotics which show activity against Gram-positive and Gram-negative bacteria, including the species *Spirochete*, *Actinomyces*, *Ricketsia* and *Mycoplasma* and have been widely used for the treatment of infectious diseases and as an additive in animal foodstuffs (Riviere & Spoo, 1995, chap. 41). They also have been applied in the treatment of honeybee to American and European foulbrood (AFB and EFB) disease, which is caused by either *Bacillus larvae* or *Streptococcus pluton*. Moreover, tetracyclines can be sprayed directly to fruit trees or other plants to treat infection by *Erwinia amylovara* and *Mycoplasma* and used to control infection of seeds by *Xanthomonas campestris*. So, the contamination of the plant with high concentrations of antibiotic implies the risk of residues into propolis (Levy, 1992). Four fused 6-membered rings, as shown in the accompanying figure (Fig. 1), form the basic structure from which the various tetracyclines are made (Stolker & Brinkman, 2005). Hence, the determination of tetracyclines as contaminants in propolis is considerably important, because of their accumulative toxicity, biological adverse effect or allergic reaction and the possibility that pathogenic organisms could become resistant to these drugs (Andersen et al., 2005).

In the United States, the maximum residue limits (MRLs) of tetracycline for all food producing species ranged from 2 to 12 mg kg^{-1} in muscle, liver, and kidney (Moats & Harik-Khan, 1995). The European Commission had set MRLs for tetracyclines – the total concentration of tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) is 100, 600, 200 and 100 μg kg^{-1} in muscle, kidney, egg and milk, respectively (Commission Regulation No. 508/99). Many methods have been developed for the determination of TCs such as microbiological assay (Kurittu, Lonnberg, Virta, & Karp, 2000), enzyme immunoassay (Heering, Usleber, Dietrich, & Martlbauer, 1998), spectrophotometry (Alwarthan, Al-Tamrah, & Sultan, 1991), flow-injection chemiluminescence methods (Pena, Palilis, Lino, Silveira, & Calokerinos, 2000), high performance liquid chromatography (HPLC) (Coyne, Bergh, & Samuelsen, 2004; Moats, 2000), and capillary electrophoresis (CE) (Nakazawa et al., 1999). Using various methods, researchers have studied trace amount of TCs in multiple matrixes

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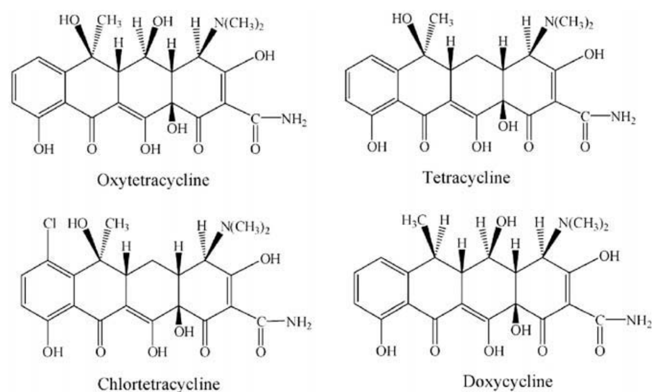

Fig. 1. Chemical structures of four tetracycline antibiotics.

(Chiayvareesajja, Chandumpai, Theapparat, & Faroongsarng, 2006; Corcia & Nazzari, 2002; Fletouris & Papapanagiotou, 2008; Huq, Garriques, & Kallury, 2006). However, there is presently no method for routine monitoring of TCs in propolis because it is considered to be an extremely complex matrix including water-soluble and fat-soluble flavonoids, aromatic acid, terpenoid compounds, wax, and pollen debris and other interfering matrix components.

The purpose of this study was to develop an effective extraction and clean-up procedure for simultaneous determination of four TCs residues in propolis. The extraction and clean-up conditions were investigated and optimised by using ultrasonic-assisted extraction and two-step SPE methods. Ultrasound can be considered a useful alternative for solid sample pretreatment because the energy imparted facilitates and accelerates some steps, such as dissolution, fusion, and leaching, among others (Luque de Castro & Da Silva, 1997). The combined usage of Oasis HLB and carboxylic acid cartridge is more suitable for purification of four TCs than other cartridges to clean-up impurities in propolis.

2. Experimental

2.1. Reagents

Methanol, ethyl acetate and acetonitrile were HPLC grade and were purchased from Baker (Deventer, Netherland). Oxalic acid, citric acid, disodium hydrogen phosphate (Na_2HPO_4), and ethylene diaminetetraacetic acid disodium salt (Na_2EDTA) were analytical reagent and obtained from Beijing Reagent Company. Pure water was purified in a Milli-Q system (Millipore, Bedford, MA, USA). Extraction buffer solution (Na_2EDTA–McIlvaine buffer, 0.1 mol L^{-1}) of propolis sample was prepared by dissolving 11.8 g of citric acid monohydrate, 13.72 g of Na_2HPO_4, and 33.62 g of Na_2EDTA in 1 L of distilled water and was adjusted to pH 4.0 ± 0.05 by dropwise addition of either 0.1 M HCl or 0.1 M NaOH. This solution was prepared weekly and stored in refrigerator until use. Oxytetracycline, tetracycline, chlortetracycline and doxycycline were purchased from Sigma (St. Louis, MO, USA). Each individual stock solution (0.1 mg mL^{-1}) was prepared by dissolving each standard of TCs in methanol and stored in the refrigerator (−18 °C). Working mixed standard solutions of these four drugs were prepared by mixing the above solutions and diluting with pure water to obtain concentrations of 5, 10, 25, 50, 100 and 200 ng mL^{-1}. These solutions can be kept under refrigeration (4 °C) for up to one week.

2.2. Apparatus and chromatographic conditions

The Waters HPLC system consisted of a 600E pump, a tunable absorbance ultraviolet detector operated at wavelength of 350 nm, a column oven, a in-line degasser, The analytical column was a Agilent ZORBAX 300SB-C18 column, 5 μm, 4.6 × 250 mm (Agilent PN 880995, USA). Oasis HLB (200 mg, 6 mL) and carboxylic acid disposal cartridge (200 mg, 3 mL) were obtained from Waters Corporation (Milford, MA, USA). About 0.01 M oxalic acid (pH 4.0), methanol and acetonitrile (70:10:20, v/v/v) were used as the mobile phase. The column oven temperature was set at 30 °C and the mobile phase was filtered, degassed and pumped at a flow rate of 1.0 mL min^{-1}.

2.3. Sample preparation

About 30 raw propolis samples were provided by beekeeper or bee product Corporation of different regions of China. The propolis powders were obtained after comminution and filtration (40 meshes) and kept at 4 °C.

2.4. Shake extraction

Aliquots of powder (2.0 g) were extracted with Na_2EDTA–McIlvaine buffer (100 mL) by using mechanical shaking in an Erlenmeyer flask at 50 °C for 4 h. Then, the solution after extraction was decanted into a 100 mL plastic centrifuge tube and centrifuged for 10 min at 5000 rpm for further clean-up.

2.5. Extraction by ultrasonic bath

Aliquots of powder (2.0 g) were placed in 100 mL beaker and the blank propolis was spiked by adding the combined standard solution of the desired concentration. They were extracted with the time of 0.5 h by 20 mL extraction buffer solution using ultrasound-assisted extraction (Kunshan Instruments Company, Kunshan, PRC; power: 100 W, frequency: 40 kHz). The temperature was controlled and maintained at 50 °C. This extraction procedure was repeated once with 20 mL extraction buffer solution. Then, the combined upper layer was decanted into a 50 mL plastic centrifuge tube and centrifuged for 10 min at 5000 rpm.

2.6. Two-step clean-up

The supernatant was poured into and flowed through the Oasis HLB cartridge equipped with reservoir with gravity, preconditioned sequentially with 5 mL methanol and 10 mL extraction buffer solution. The cartridge was washed with 10 mL of 5% methanol (methanol/pure water = 5/95; v/v) and then dried completely by sucking air through vacuum pump. Finally, analytes were eluted from the Oasis HLB cartridge with 15 mL ethyl acetate.

The ethyl acetate eluate was transferred into a carboxylic acid cartridge, previously conditioned 5 mL ethyl acetate. The cartridge was washed with 10 mL methanol and then dried to remove solvent and nonspecifically adsorbed impurity by sucking air about 5 min. The carboxylic acid cartridge was eluted with 4 mL mixture of 0.01 M oxalic acid (pH 4.0) and acetonitrile (6:4, v/v), and the eluate was evaporated to 2 mL volume under a stream of nitrogen at 45 °C. The final extract was filtered through a 0.2 μm nylon filter directly into a high performance liquid chromatography vial.

3. Results and discussion

3.1. Optimisation of chromatographic conditions

For tetracyclines, there are two maximum absorption wavelengths at 275 and 350 nm in ultraviolet detector. Detection wavelengths of 275 and 350 nm were tested to monitor the extracting solutions of propolis in this study. Though both of them have high response, less interfering compounds were observed at 350 nm in

the chromatogram of propolis. So, 350 nm was selected as the optimum detection wavelength.

The columns including Symmetry C18, Novapak C18, μBondpak C18 and Agilent ZORBAX SB-C18 were tried to separate four TCs. After many trials, TCs can be separate by using μBondpak C18 and Agilent ZORBAX SB-C18 column. But, peak tailings and wide peak of OTC, TC, CTC and DC occurred with μBondpak C18 column. In order to optimise the separation and get better signal of peaks, Agilent ZORBAX SB-C18 was tested and symmetrical and sharp peaks without tailings or overlaps were obtained by using it.

The TCs with metal ions and adsorb on the silanol group is easy to form chelate complexes in a reversed-phase column because of the presence of two ketone groups, so that TCs are apt to appear as tailing peaks (Stolker & Brinkman, 2005). In order to avoid forming chelate complexes and their adsorption on reverse phase columns, various acids were selected and symmetrical non-tailed peaks were obtained when using oxalic acid. The oxalic acid as ionisation suppression agent can minimise the occurrence of mixed separation mechanisms for TCs antibiotics and effectively mitigate incomplete ionisation due to effect of residual silanols on the stationary phase. The oxalic acid concentration had an important influence on peak symmetry and baseline, so decrease in tailing factors and no baseline drift were observed when the concentration was increased up to 0.01 M in pure water solution. Acetonitrile–0.01 M oxalic acid (pH 4.0) and methanol–0.01 M oxalic acid (pH 4.0) as mobile phase were tried to separate the four tetracyclines in reverse phase column, but the complete separation of four tetracyclines cannot be obtained at any proportion of acetonitrile–0.01 M oxalic acid and the peak became more wider at any proportion of methanol–0.01 M oxalic acid. So, 0.01 M oxalic acid (pH 4.0), methanol and acetonitrile (70:10:20, v/v/v) was confirmed as the optimum combination of mobile phase, because the better resolutions and satisfactory peak shape of four tetracyclines were obtained in the chromatogram within run time of 18 min.

3.2. Optimising extraction and clean-up

3.2.1. The selection of extraction condition

In order to obtain optimal extraction efficiency, extraction solvents and extraction time were investigated. Various solvents including methanol, acetonitrile, ethyl acetate and Na_2EDTA–McIlvaine buffer were tested for the extraction of propolis sample. Methanol, acetonitrile, and ethyl acetate were the effective solvent of propolis, which resulted in the co-extraction of lots of fat-soluble flavonoids, terpenoid compounds, wax as well as pollen debris from propolis. The chromatograms obtained for propolis using the extract from methanol, acetonitrile, or ethyl acetate presented many interfering peaks, which overloads the column. Na_2EDTA was included in extraction solution to avoid the interference from metals in the process of extraction. Citrate was chosen as one of constituents of extraction buffer as it provided better recoveries of TCs. So, Na_2EDTA–McIlvaine buffer as the extraction solvent was the better choice, because the satisfactory recovery was obtained and less impurity was found.

Shake extraction is widely used although its disadvantages include time consumption and low recovery. In this work, the poor recoveries (<30%) were sourced by using shake extraction (Table 1). It is difficult to resolve propolis matrix with Na_2EDTA–McIlvaine buffer though this buffer was effective solvent of TCs, so it is necessary to select a new way to extract TCs through propolis. Ultrasonic extraction was applied as a simple, inexpensive method applicable to a wide range of animal tissues and environmental samples. In propolis analysis, sonication was used in sample pretreatment for better dissolution of propolis. The most important purpose of optimisation experiments was to improve the extraction efficiency, achieved by changing the time (min), temperature (°C) and amount of extraction solution (mL), with minimum solvent consumption and minimum duration of the extraction time and results are shown in Fig. 2. The compared results of recoveries between shake extraction and ultrasonic-assisted extraction are listed in Table 1. In the final, the recovery of TCs obtained by ultrasonic extraction was better than that obtained by shake extraction.

Table 1
Effect of different extraction method and solid phase extraction columns on the recovery of TCs ($n = 5$).

Extraction method	Recovery (mean ± SD%)			
	OTC (200 ng g^{-1})[a]	TC (200 ng g^{-1})	CTC (300 ng g^{-1})	DC (300 ng g^{-1})
Shake extraction	25.1 ± 14.7	17.4 ± 11.9	ND[b]	ND
Ultrasonic-assisted extraction	82.9 ± 6.09	74.1 ± 9.25	63.9 ± 11.8	65.2 ± 12.4
C18	61.0 ± 19.4	59.1 ± 18.1	48.1 ± 24.9	50.3 ± 23.7
Oasis HLB	171.4 ± 3.91	167.9 ± 4.03	133.1 ± 6.91	128.3 ± 8.49
C18 + carboxylic acid	47.2 ± 13.1	35.5 ± 12.7	29.3 ± 19.7	21.9 ± 22.5
Oasis HLB + carboxylic acid	81.2 ± 5.74	74.0 ± 9.31	67.6 ± 10.8	65.3 ± 11.9

[a] Spiked concentration of TCs in propolis samples.
[b] ND; not detected.

3.2.2. The selection of clean-up condition

C18, Oasis HLB and carboxylic acid disposable cartridges were tested for the clean-up of propolis samples. However, no success was obtained for single one of them because of the interference of impurities and extraneous substances. The poor recoveries, repeatability and reproducibility were obtained by the clean-up of generic C18. The Oasis HLB, made of lipophilic and hydrophilic copolymer with a balanced ratio of two monomers, was chosen for the clean-up of propolis sample. TCs can be retained on this polymer by both polar and non-polar interactions, which allow better recoveries. The recoveries obtained for OTC, TC, CTC and DC exceeded to 120% and more impurities were found, which mean that only utilisation of Oasis HLB failed to effectively purify the impurity of propolis, so an additional solid phase extraction (SPE) clean-up step was then added (Bernardete, Ana Luiza, Marcus Antonio, & Mychelle, 2007). Co-extraction with two-step SPE was tried for clean-up of propolis. TCs can interact with hydrogen ions to form cations under acidic condition. Extracts containing TCs may be cleaned up by solid phase extraction on cartridges packed with weak cation-exchange (WCX) packing. The cartridge can then be washed by solvents, such as acetonitrile, acetone, methanol to remove impurities that could affect subsequent determinations. TCs may also be eluted from a WCX cartridge using a solvent with a low pH to cancel the charge on the carboxyl groups of the stationary phase (George et al., 2005). The compared results obtained by using different columns and their series connection are shown in Table 1.

3.3. Validation of method

3.3.1. Specificity

The chromatographic interference from endogenous compounds was assessed by comparing chromatograms of blank propolis and propolis spiked with four TCs. An ultraviolet detector was used to check for the presence of coeluting peaks of four TCs. Fig. 3 represents chromatograms of four TCs from propolis after two-step SPE extraction along with blank propolis sample. No interference of endogenous peaks with OTC, TC, CTC and DC at their respective retention times (OTC; 4.42 min, TC; 4.91 min, CTC; 9.28 min and DC; 14.05 min) in blank propolis were detected.

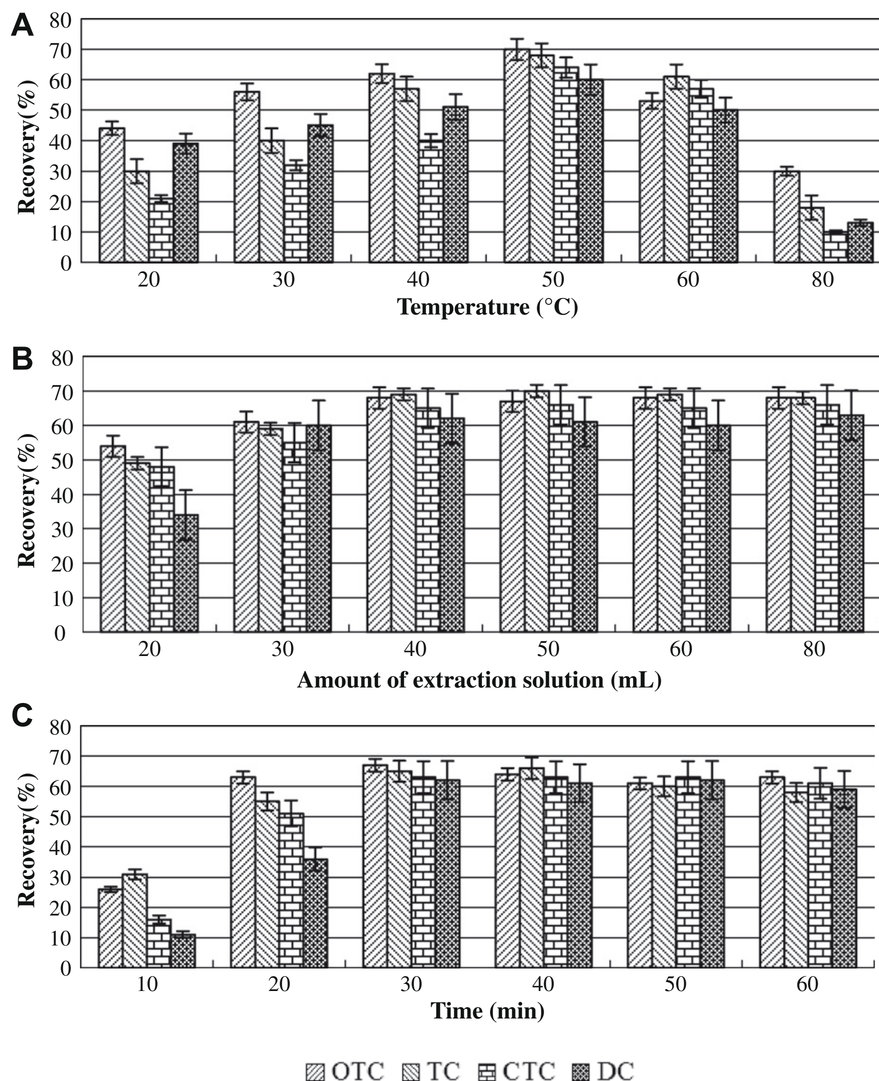

Fig. 2. Effect of time (A), temperature (B) of sonication and (C) amount of extraction solution on the recovery of OTC, TC, CTC and DC.

3.3.2. Method linearity and sensitivity

Calibration curves were obtained separately by plotting the peak area of four TCs to the spiked TCs theoretical concentrations in blank propolis on the same day ($n = 5$) and on different days ($n = 4$). The linearity of calibration curve was evaluated by analysis of six different concentrations. The calibration curves of fortified samples showed acceptable linear response ($R^2 > 0.99$) in the concentration of 100–5000 ng g^{-1} for four TCs in 20 replicates of six concentrations, respectively. The mean (±SD, RSD) slope and intercept of the calibration curves for four TCs are listed in Table 2. The analysis of variance (ANOVA) with a lack-of-fit test was performed to validate the regression data (Statgraphics-Plus V. 5.1). This test allows determination of whether the selected model is adequate to describe the observed data, or whether a more complicated model should be used. The test is performed by comparing the variability of the proposed model residuals to the variability between observations (chromatographic response) at replicate values of the independent variable (known concentration of compounds in the sample solutions). The results of *F*-ratio and *P*-values obtained in the calibration range were estimated. For four TCs, *P*-values were greater than 0.05 for all compounds and linear regression models were thus adequate for the obtained data at a confidence level of 95%, as we have seen from Table 2.

Limit of quantification, which is the minimum concentration of TCs that can be quantitatively determined with a peak area to base line ratio of at least 10:1. The LOQ was 100 and 150 ng g^{-1} for OTC, TC and CTC DC, respectively, determined by analysing matrix samples spiked with known concentration of analyte.

3.3.3. Precision and accuracy

In order to assess the intra- and inter-day precision and accuracy of the assay, propolis samples at low, medium and high concentrations were prepared as described above. The intra-day precision of the assay was assessed by calculating the coefficients of variation (CV) for the analysis of propolis samples in quintuplicate; and inter-day precision was determined by the analysis of propolis samples on four consecutive days. Accuracy was calculated by comparing the averaged measurements and the nominal values (Table 3). The criteria for acceptability of precision were that the relative standard deviation (RSD) for each concentration level should not exceed 15% with the exception of the LOQ, where it should not exceed 20%. Similarly, for accuracy, the averaged value should be within 15% of the actual value except at LOQ, where it should not deviate by more than 20%, according to the regulation of Food and Drug Administration (http://www.fda.gov/CDER/GUIDANCE/4252fnl.htm). The deviation of the mean from the true value serves as the measure of accuracy.

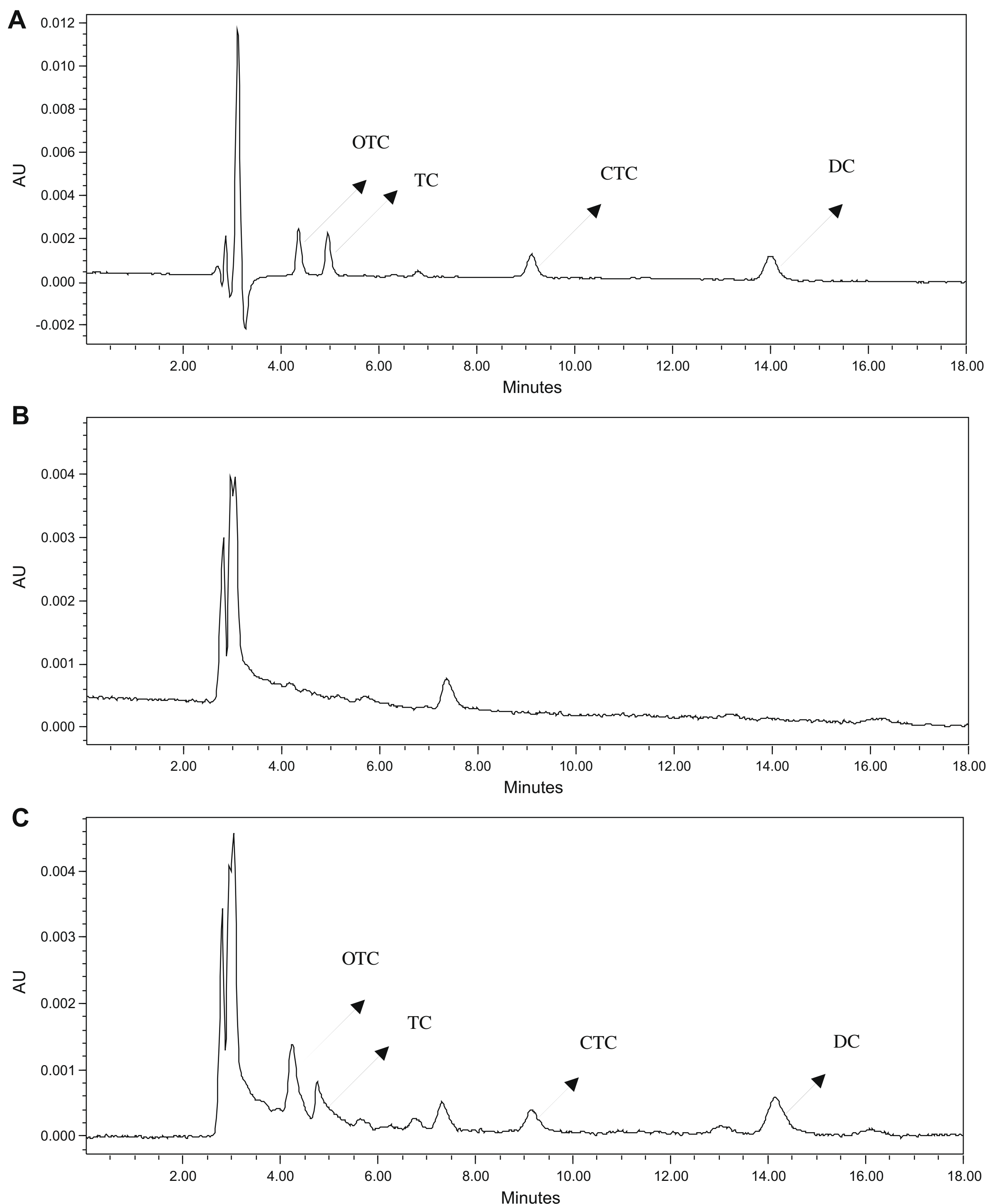

Fig. 3. HPLC chromatograms obtained by UV detection at 350 nm (A) standards, in blank propolis extraction, at 150, 150, 200 and 200 ng g^{-1} for OTC, TC, CTC and DC, respectively; (B) blank propolis sample extraction; (C) spiked blank propolis at 100, 100, 150 and 150 ng g^{-1} for OTC, TC, CTC and DC, respectively.

3.3.4. Recovery

Absolute recoveries of four TCs were evaluated by spiked blank propolis samples at three concentrations from 100 to 500 ng g^{-1} in sextuplicate. It was calculated by comparison of peak areas from spiked samples with the standard solutions of equivalent concentrations. The mean extraction recoveries of four TCs were in the range of 61.9–88.5% and the RSDs was between 4.80% and 13.2% for propolis samples (Table 3). Therefore, the proposed method was applicable for the determination of TCs residues in propolis. There was a variation between the recoveries for four analytes, it is because the recovery is possibly related with the structure of analyte and its combinative intensity with propolis.

Table 2
Analytical parameters of OTC, TC, CTC and DC.

Regression equation[a]	OTC	TC	CTC	DC
Linear dynamic range (ng g^{-1})	100–5000	100–5000	150–5000	150–5000
Slope	236.82	171.62	172.67	325.86
Sa	17.1	24.5	17.7	36.9
RSD[b] (%)	7.00	10.1	7.23	13.7
Intercept	1655.9	1422.6	3922.3	6318.6
Sb	27.2	32.5	30.3	40.3
RSD[c] (%)	11.1	12.9	11.8	14.6
F-ratio[d]	0.73	0.79	0.82	0.97
P-value[e]	0.4952	0.4364	0.4498	0.4136
Correlation coefficient (R^2)	0.9992	0.9990	0.9968	0.9947
Limit of quantification (LOQ, ng g^{-1})	100	100	150	150

Sa, standard deviation of slope; and Sb, standard deviation of intercept.
[a] Linear regression analysis with a regression equation of $y = ax + b$, in which x is the concentration in ng g^{-1} and y is the peak area.
[b] Relative standard deviation of slope.
[c] Relative standard deviation of intercept.
[d] *F*-ratio for lack-of-fit test.
[e] Probability value for lack-of-fit test.

Table 3
The intra- and inter-day accuracy, precision and recovery of propolis samples.

Spiked concentration (ng g^{-1})	Intra-day ($n = 5$)		Inter-day ($n = 4$)		Recovery (%) ($n = 6$)	RSD (%)
	Measured concentration (ng g^{-1})	RSD (%)	Measured concentration (ng g^{-1})	RSD (%)		
Oxytetracycline						
100	103.6 ± 3.77	3.64	102.4 ± 9.10	8.89	71.9 ± 6.20	8.62
200	201.3 ± 4.81	2.39	200.4 ± 12.8	6.39	79.8 ± 4.71	5.90
500	497.2 ± 5.12	1.03	499.1 ± 29.7	5.95	87.6 ± 4.25	4.85
Tetracycline						
100	109.1 ± 4.63	4.24	98.7 ± 10.9	11.0	74.2 ± 7.94	10.7
200	205.8 ± 5.35	2.60	207.1 ± 15.7	7.58	70.9 ± 6.71	9.46
500	481.5 ± 5.97	1.24	501.3 ± 31.6	6.30	88.5 ± 4.25	4.80
Chlortetracycline						
150	147.9 ± 5.09	3.44	155.1 ± 18.7	12.1	61.9 ± 8.16	13.2
300	299.3 ± 8.64	2.89	291.1 ± 30.1	10.3	70.7 ± 8.25	11.7
500	500.8 ± 16.3	3.37	487.9 ± 41.8	8.57	79.8 ± 4.96	6.22
Doxycycline						
150	142.5 ± 5.37	3.77	148.7 ± 17.1	11.5	64.1 ± 8.35	13.0
300	304.9 ± 9.35	3.07	307.1 ± 34.6	11.3	68.7 ± 8.71	12.7
500	489.5 ± 17.9	3.66	497.2 ± 65.9	13.2	78.4 ± 7.18	9.16

3.3.5. Stability

The stability studies evaluate the stability of the trace amount of TCs residue of propolis and the stability of aqueous standard solutions was also evaluated.

3.3.5.1. Standard solutions stability. The solutions were analysed every two weeks and were compared with freshly prepared standards (200 ng mL^{-1}) in the darkness. The standard solutions were found to be stable for three months when refrigerated at −18 °C, but about half degraded at 2–8 °C for three months and one-third of four TCs were found at room temperature for one month. However, all of the four TCs were not found at room temperature for one week without photophobic condition.

3.3.5.2. Processed samples stability. The samples reconstituted with mobile phase were found to be unstable for over 24 h at room temperature without photophobic condition. The TCs of extractant was unstable over one month when refrigerated at −18 °C, but about 80% of them degraded at 2–8 °C for two weeks in the darkness. The most likely reason is that the TCs were easy to degrade under the acid condition and sensitive to light.

3.4. Application of the method

The method was applied to real propolis samples provided by some beekeepers and supermarkets. In two out of 30 analysed samples, traces of tetracyclines were found and treatment to control AFB or EFB with TCs was substantiated by the beekeeper. Result indicated that the residue concentrations in propolis were 108 ± 6.6 ng g^{-1} (No. 9) and 143 ± 4.7 ng g^{-1} (No. 24) for OTC and TC, respectively.

4. Conclusion

In this paper, an efficient method for simultaneous determination of four TCs residue in propolis, using HPLC-UV on the basis of ultrasonic-assisted extraction and two-step solid phase extraction, has been developed for the first time. Good recoveries (61.9–88.5%) and RSDs (<15%) were obtained, with limits of quantification, ranging from 100 to 150 ng g^{-1}. Levels of OTC and TC in propolis samples were successfully determined using the method. It is the fundamental work to establish more convenient and simple method for the determination of TCs in propolis.

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