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Journal of Chromatography A, 1022 (2004) 125–129
JOURNAL OF CHROMATOGRAPHY A
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Liquid chromatography with ultraviolet absorbance detection for the analysis of tetracycline residues in honey

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Received 12 May 2003; received in revised form 18 July 2003; accepted 23 September 2003

Abstract

The separation of tetracyclines (TCs) using reversed-phase liquid chromatography (LC) is proposed. The use of an amide-based stationary phase prevents the interaction of tetracyclines with the residual silanol groups and thus avoids the appearance of tailed peaks. Detection was based on using an UV spectrophotometer and gradient elution with acetonitrile–oxalic acid as mobile phase permitted good separation of all the peaks. Specificity was demonstrated by the retention characteristics, UV spectra and peak purity index. Linearity, precision, recovery and sensitivity were satisfactory. The procedure was applied to the analysis of tetracycline residues (tetracycline, oxytetracycline (OTC), chlortetracycline (CTC), doxycycline (DC), minocycline (MINO) and methacycline (MTC)) in honey of different types. Extraction involved using a mild acidic solvent containing EDTA to release protein-bound or sugar-bound tetracyclines. For the clean-up step, solid phase extraction using phenyl cartridges was applied. Detection limits in the honey using the proposed procedure are between 15 and 30 ng g^{-1}, depending on the tetracycline.

Keywords: Food analysis; Honey; Tetracycline; Antibiotics

1. Introduction

Tetracyclines (TCs) are broad-spectrum antibiotics which show activity against Gram-positive and Gram-negative bacteria, including some anaerobes [1] and have been widely used for the treatment of infectious diseases and as an additive in animal foodstuffs. In 1990, the Commission of the European Union laid down the procedure for establishing maximum residue limits (MRLs) of veterinary drugs in foodstuffs of animal origin. However, no MRLs have been fixed for use with bee products. Antibiotics such as tetracyclines have had MRLs imposed for their use in large animals but are illegal for use with bees. Honey is composed primarily of sugars and water [2] being a natural healthy food. At present, application of the law in relation to these antibiotics is not harmonised across all member states. Some countries do not permit honey to contain drugs above the corresponding detection limit, while others apply an action level such as 50 ng g^{-1}. However, it must be stressed that the presence of these drugs in honey is illegal at any level [3]. The Spanish plan for residue control and healthy food (Plan CREHA) of 2002 has established maximum limits for the concentration of residual substances [4]. As regards the group of tetracyclines, the analytes included are tetracycline, oxytetracycline (OTC) and chlortetracycline (CTC), which have been analyzed in meat, milk, honey and eggs. The detection limit obtained using liquid chromatography (LC) is 20 ng g^{-1} or 20 ng ml^{-1} and the maximum acceptable limit has been set at 100 ng g^{-1} or 100 ng ml^{-1}. These limits are similar to those marked by the Belgian Agency for Safe Foods.

Tetracyclines can be successfully determined using liquid chromatography (LC) in the reversed-phase mode [5–7] and with different detection modes, such as spectrophotometry [8–13], fluorescence [14,15], fast-atom-bombardment mass spectrometry (FAB-MS) [16] and MS–MS [17,18].

In the present study, the separation of tetracycline, oxytetracycline, chlortetracycline, doxycycline (DC), minocycline (MINO) and methacycline (MTC) was optimized using an UV detector and a stationary phase involving a ligand with amide groups and the endcapping of trimethylsilyl. This phase also proved satisfactory for the determination of fluoroquinolone antibacterial agents [19]. These tetracyclines

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doi:10.1016/j.chroma.2003.09.066

were selected because OTC, TC, CTC and DC are commonly applied to food-producing animals including honeybees, MINO has the highest biological activity and MTC is more stable than TC. The procedure is applied to the analysis of tetracycline residues in different types of honey.

2. Experimental

2.1. Apparatus

The LC system consisted of a Shimadzu FCV-10ALvp (Shimadzu, Kyoto, Japan) liquid chromatograph operating at room temperature with a flow rate of 1 ml min^{-1}. The solvents were degassed using a membrane system Shimadzu DGU-14A. The spectrophotometric detector was a photo-diode array Shimadzu SPD-M10Avp operating at wavelengths of 270 and 355 nm for all tetracyclines. The software was Class-LC10 (Shimadzu) and the detector was connected to a SPD-MXA integrator. Aliquots of 100 μl were injected manually using a Model 7125-075 Rheodyne injection valve (Rheodyne, Berkeley, CA, USA). The analytical column used for the reversed-phase technique was packed with Discovery RP-Amide C_{16} with a particle size of 5 μm (Supelco, Bellefonte, PA, USA). A guard column packed with the same stationary phase was also used. Stirring of the samples was carried out using a IKA KS 130 basic vibratory stirrer (IKA, Germany). A Büchi vacuum V-500 rotatory evaporator R-200 coupled to a Büchi heating bath B-490 (Switzerland) and a V-800 vacuum controller was used to concentrate the organic sample extracts. For solid phase extraction, a Visiprep-DL vacuum manifold (Supelco, Bellefonte, PA, USA) was employed and different type of cartridges (Discovery DSC-18, DSC-18Lt, DSC-8, DSC-Ph and DSC-CN SPE) with 500 mg and 3 ml were compared.

2.2. Reagents and samples

Acetonitrile, methanol and ethyl acetate (Lab Scan, Dublin, Ireland) were of liquid chromatographic grade. Doubly distilled water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). The 10 mM oxalic acid solution of pH 3 was prepared daily from the commercial product (Panreac, Barcelona, Spain) by dissolving 1.26 g of oxalic acid dihydrate en 1 l of distilled water and adjusting pH by adding dropwise 10 M potassium hydroxide (Panreac). Stock solutions (1000 μg ml^{-1}) of TC, OTC, CTC, DC, MINO (Sigma, St. Louis, MO, USA) and MTC (Riedel-de Haën, Seelze, Germany) were prepared by dissolving 10 mg of the commercial products, without previous purification, in 10 ml of methanol. They were kept in dark bottles in the freezer at less than 0 °C and were stable for at least 3 months. Working standard solutions were prepared daily by dilution with the oxalic acid solution. The McIlvaine buffer (pH 4.0 ± 0.05) and McIlvaine buffer–EDTA solutions were weekly prepared as previously described [20].

The samples of different types of honey (thousand flowers, acacia, orange, eucalyptus and honeydew) were obtained commercially and from several beekeepers.

2.3. Analytical procedure

A sample of 3 g of honey was weighed into a polypropylene tube and dissolved in 6 ml of 0.1 M Na_2EDTA–McIlvaine buffer (pH 4). The solution was stirred for 10 min using a vibratory stirrer at 800 rpm. The sample solution was applied to a Discovery DSC-phenyl SPE (500 mg) cartridge activated with 5 ml of acetonitrile and 5 ml of the 10 mM oxalic acid solution (pH 3) and conditioned with 5 ml of a saturated Na_2EDTA solution. The phenyl cartridge containing the sample was washed with 5 ml of the oxalic acid solution. TCs were eluted with 5 ml of 10% (v/v) methanol in ethyl acetate and the first 0.5 ml of the eluate were discarded. The eluate was concentrated under vacuum at 240 bar and 40 °C and the residue was dissolved in 0.5 ml of the oxalic acid solution. Aliquots were filtered through a 0.45 μm nylon Millipore chromatographic filter and injected into the chromatograph.

3. Results and discussion

3.1. Selection of the mobile phase for using the amide-based column

Tetracyclines form complexes with metal ions and adsorb on the silanol groups in the reversed phase [6]. To prevent this process, which produces tailing peaks, we selected a stationary phase with the endcapping of trimethylsilyl. Several acids were used for the mobile phase, and symmetrical non-tailed peaks were obtained when using oxalic acid. The optimal mobile phase was selected by varying the proportion of ACN, the concentration of oxalic acid and pH. The retention factors of TCs decreased when the proportion of organic solvent was increased and at higher pH values. The oxalic acid concentration had an important influence on peak symmetry, a strong decrease in tailing factors was observed when the concentration was increased up to 10 mM. Isocratic elution was not possible since the TCs presented a very different retention behavior. The optimal mobile phase for separating MINO, OTC and TC (containing 5–10% (v/v) ACN) did not elute the rest of the compounds. For higher ACN percentages, TCs eluted together at the void time. Then, a gradient elution technique was tried. The optimal gradient was found to be an initial isocratic step with a 88:12 (v/v) 10 mM oxalic acid (pH 3):ACN mixture during 9 min, a gradient to 82:18 (v/v) 10 mM oxalic acid (pH 3):ACN mixture in 0.01 min and, finally, this mixture was maintained for 16 min. The initial conditions were re-established in 1 min and held for 15 min. The chromatogram

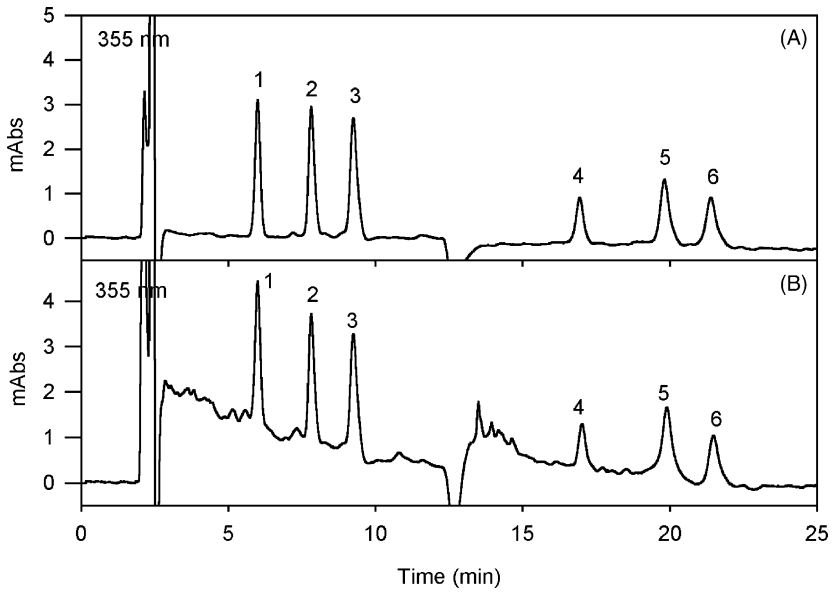

Fig. 1. Chromatographic profiles using the amide-based column and gradient elution. Flow rate, 1 ml min^{-1}; injected sample, 100 μl. (A) Standards mixture containing 500 ng ml^{-1} of each TC. The peaks correspond to: 1, MINO (t_R = 6.0 min); 2, OTC (7.8 min); 3, TC (9.3 min); 4, CTC (17.1 min); 5, MC (20.0 min) and 6, DC (21.6 min). (B) Honey extract fortified with 500 ng ml^{-1} of each TC.

obtained using this gradient elution program is shown in Fig. 1A.

3.2. Calibration, detection limits and repeatability

Calibration graphs were performed by the external standard technique by plotting concentration (ng ml^{-1}) against peak area. Table 1 shows the equations obtained for the calibration graphs and the regression coefficients at 355 nm. The statistical analysis of the linear regression graphs shows that there is no statistically significant difference between the intercept and zero ($P = 0.224$ for MINO, $P = 0.375$ for OTC, $P = 0.339$ for TC, $P = 0.056$ for CTC, $P = 0.248$ for MC and $P = 0.148$ for DC) at the 95% confidence level. The repeatability of the method was calculated using the average relative standard deviation (R.S.D.) for ten replicate injections of the same sample at 300 ng ml^{-1} level. The reproducibility was calculated using the R.S.D. for ten injections of the same sample at 300 ng ml^{-1} level on different days during the method development. The detection limits were calculated on the basis of 3σ (σ being the residual standard deviation of the intercept) using the regression lines for the standards. Values are also given in Table 1.

3.3. Extraction and clean-up procedures

For the determination of tetracyclines in honey, a previous extraction step need to be optimized. TCs bind with proteins and, consequently, extraction was carried out using a mild acidic solvent containing EDTA. A buffer 0.1 M EDTA–McIlvaine (pH 4) was selected [6]. The chromatograms obtained for honey using the extract from the EDTA–McIlvaine buffer presented many interfering peaks, corresponding to substances which might have originated a matrix effect. Thus, a clean-up step using solid-phase extraction with cartridges which can separate TCs from impurities was an important variable. The optimization of the clean-up procedure was as follows: a sample of 3 g of honey was weighed into a polypropylene tube, fortified with TCs at concentrations of 500 ng g^{-1} and dissolved in 6 ml of 0.1 M Na_2EDTA–McIlvaine buffer (pH 4). The solution was stirred for 10 min using a vibratory stirrer at 800 rpm. The

Table 1
Calibration characteristics of tetracyclines

Tetracycline	Intercept	Slope (ml ng^{-1})	Correlation coefficient	Linearity (ng ml^{-1})	DL (ng ml^{-1})	Repeatability R.S.D. (%, $n = 10$)	Reproducibility R.S.D. (%, $n = 10$)
MINO	−568 ± 441	194 ± 1.2	0.9996	50–650	7	2.2	2.5
OTC	−520 ± 562	189 ± 1.4	0.9994	50–650	9	1.5	3.0
TC	−361 ± 261	215 ± 0.9	0.9997	50–650	4	1.2	2.1
CTC	−530 ± 251	99 ± 0.6	0.9998	100–850	8	3.2	4.0
MC	−300 ± 207	166 ± 0.6	0.9999	100–850	4	2.5	4.8
DC	−534 ± 343	104 ± 0.7	0.9998	100–850	10	3.2	3.5

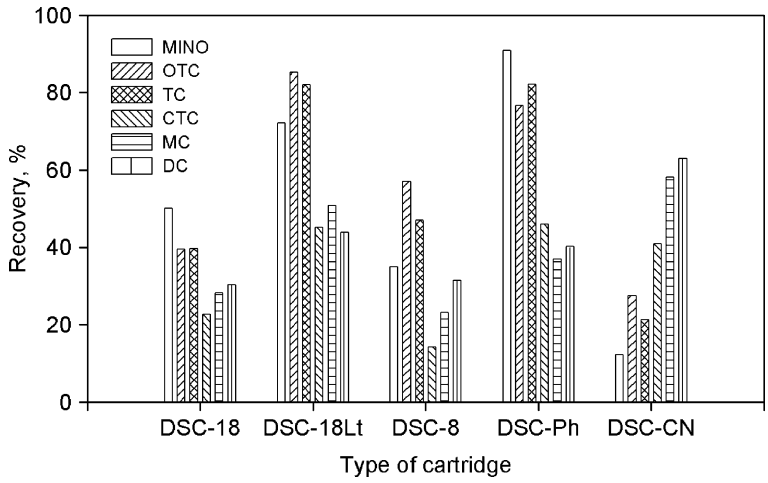

Fig. 2. Comparison of the elution graphs for TCs from a fortified honey sample by using different type of SPE cartridges.

variables optimized for the solid phase extraction were: the nature of the clean-up cartridge, the influence of a washing step, the optimal solvents and the volume for eluting TCs from the cartridge, and the final concentrating of the sample.

Different type of SPE Discovery reversed-phase cartridges with 500 mg and 3 ml were compared. The cartridges used were DSC-18 (octadecyl, 18% C), DSC-18Lt (octadecyl, 11% C), DSC-8 (octyl, 11% C), DSC-Ph (phenyl, 7% C) and DSC-CN (cyanopropyl, 7% C). The cartridges were previously activated with 5 ml of ACN and 5 ml of the 10 mM oxalic acid solution (pH 3) and conditioned with 5 ml of a saturated Na_2EDTA solution to prevent the formation of chelates between the TCs and the metal ions and a strong interaction with the stationary phase. The honey was passed through the cartridge and this was washed with 5 ml of the oxalic acid solution. TCs were eluted using 5 ml of the chromatographic mobile phase. Fig. 2 shows the elution graphs obtained for all TCs when using the different type of cartridge. Very low recoveries were obtained for the cartridges containing cyano groups and low recoveries for the DSC-8 and DSC-18. Best results were obtained with the DSC-18Lt and DSC-Ph, which provided high recoveries for MINO, OTC and TC and lower values for the other TCs, which remained partially retained.

It was proved that TCs were well retained in the cartridge and were not eluted in the washing step, so the solvents and the optimal volume for eluting TCs were studied. Table 2 shows the recovery values obtained when using different mixtures of acetonitrile, oxalic acid, ethyl acetate and methanol. Best recoveries were obtained for all solvents using the DSC-Ph cartridges, which was therefore selected. The mixtures of ACN with oxalic acid gave low recoveries and higher ACN percentages cannot be used because the peaks appeared as split. The use of ethyl acetate and methanol mixtures led to the best results and high recovery values were obtained using a 90:10 mixture. The volume of this solvent used was varied in the 4–10 ml range and good values were obtained up to 5 ml, which was the volume selected. The possibility of discarding the first fraction of the eluate was assayed in an attempt to eliminate matrix interferences. When discarding 0, 0.25, 0.50, 0.75 and 1.0 ml of eluate, the recoveries obtained were 85.8, 89.9, 93.7, 67.0 and 57.5, respectively. As can be seen, recovery values remained practically constant when the first 0.25 and 0.5 ml from the eluate were discarded but significantly lower recoveries were obtained for higher discarded volumes. Thus, the procedure includes discarding the first 0.5 ml.

Table 2
Solvents used for eluting tetracyclines from SPE cartridges

Solvent/TC	Recovery using DSC-Ph/DCS-18Lt cartridges					
	MINO	OTC	TC	CTC	MC	DC
Acetonitrile	0/0	0/0	0/0	0/0	0/0	0/0
Acetonitrile:oxalic acid (20:80)	84/81	95/90	99/85	87/78	82/69	74/58
Acetonitrile:oxalic acid (12:88)	91/72	77/86	82/82	46/45	37/51	40/44
Ethyl acetate	63/39	62/25	49/24	44/17	22/12	57/7
Ethyl acetate:methanol (95:5)	78/57	87/67	78/72	56/34	60/42	55/39
Ethyl acetate:methanol (90:10)	97/78	91/77	83/82	81/66	72/64	91/68
Ethyl acetate:methanol (80:20)	95/73	91/77	83/81	79/65	74/67	91/66

Finally, concentrating the sample eluted from the cartridge to a small volume was assayed in order to decrease TCs detection limits. The optimal conditions for this step were to evaporate to dryness the 5 ml eluate volume under vacuum at 240 bar and 40 °C and to dissolve the residue in 0.5 ml of the oxalic acid solution.

3.4. Analysis of honey samples: recovery studies

The procedure was applied to the analysis of tetracycline residues in different types of honey. A recovery study was carried out by the standard addition technique, by spiking five samples of different types of honey with the TC standards prior to applying the extraction procedure at levels between 15–30 ng g^{-1}. A chromatographic profile of a honey extract fortified with TC is shown in Fig. 1B. Data of average recovery and relative standard deviation, R.S.D. ($n = 6$), for the different TCs in the honey samples were—thousand flower, mean recovery: 94.7; R.S.D.: 4.3; acacia: 92.1, 4.7; eucalyptus: 96.1, 5.4; honeydew: 92.5, 4.5 and orange: 94.2, 4.0. These results demonstrated that recoveries were almost quantitative. A two way ANOVA test was then used to evaluate the existence of statistically differences, using the Tukey test to carry out all the pairwise multiple comparisons. The difference in the mean values among the levels of the factor type of honey show that there is not a statistically significant difference ($F = 0.943$, $P = 0.460$). For the factor tetracycline, there was a statistically significant difference ($F = 20.338$, $P < 0.001$), TC recovery being lower than the other TCs.

The chromatographic peaks were identified by comparing the retention data obtained for the standards and the sample spiked with the standards under identical conditions and using the photo-diode array detector to continuously measure the UV-Vis spectrum while the solute passed through the flow cell. The peak purity curves and chromatogram ratios obtained using two wavelengths for the peaks corresponding to the TCs added to the honey samples showed that no impurities coeluted with the analytical peaks. The averaged values (mean ± S.D.) for the purity index were: MINO, 0.8902 ± 0.0578 ($n = 5$); OTC, 0.9167 ± 0.0670 ($n = 5$); TC, 0.9215 ± 0.0370 ($n = 5$); CTC, 0.9160 ± 0.0300 ($n = 5$); MC, 0.9589 ± 0.0356 ($n = 5$) and DC, 0.9213 ± 0.0395 ($n = 5$).

Finally, different honeys were analyzed and no TCs were identified in any of the samples above the detection limits. Detection limits in the honey using the proposed procedure are between 15 and 30 ng g^{-1}, depending on the tetracycline.

4. Conclusion

The procedure can be applied successfully to the analysis of tetracycline residues using reversed-phase liquid chromatography with UV detection and gradient elution. The use of an Amide-C_{16} column with the endcapping of trimethylsilyl was satisfactory to prevent interaction of TCs with the silanol groups of the stationary phase, thus avoiding the appearance of tailing peaks. Selection of SPE cartridges for the method is critical, best results being obtained using phenyl cartridges. The recovery study carried out in several honey samples led to good results, detection limits in the honey ranging between 15 and 30 ng g^{-1}, depending on the tetracycline.

Acknowledgements

The authors are grateful to the Spanish INIA (Project CAL01-025) and to Comunidad Autónoma de la Región de Murcia (CARM, Fundación Séneca, Project PB/35/FS/02) for financial support.

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