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Multiresidue chromatographic method for the determination of antibiotic residues in honey by high-performance liquid chromatography with DAD detection

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Abstract: Clandestinely, consumers may be exposed to antibiotic (ATB) residues in honey, which could pose a health concern. For the first time, the simultaneous determination of Florfenicol (FF), Penicillin G (PG), and Tetracycline is described in this paper. The multiresidual method was developed and optimized using high-performance liquid chromatography (HPLC) coupled to a diode array detector (DAD). These ATBs were separated on a C18 analytical column after a cleanup process followed by solid-phase extraction (SPE). For the first time, the chromatographic conditions were perfected. After the method validation process, the method was used to assess ATB residues in four Lebanese honey samples. ATBs were separated in less than 15 min with an isocratic elution using a mixture of 80 % potassium dihydrogen phosphate aqueous solution, 20 % acetonitrile. The UV detection was performed at 350 nm for TC, 224 nm for FF, and 230 nm for PG. The proposed method was linear ($R^2 \geq 0.996$) within the concentration ranges of 0.7-17.5 mg.Kg^{-1} for the three compounds. Both intra- and inter-day precision, expressed as RSD, were ≤15 %. The method was subsequently successfully applied to analyze examined ATB residues in honey samples collected from Lebanese beekeeping. The method described could be a valuable tool to conduct a comprehensive survey of honey samples produced in Lebanon, especially in the lack of serious national oversight.

Keywords: Multiresidue, Antibiotics, HPLC-DAD, Honey, SPE.

1. Introduction

Honey is a natural sweet food, produced by *Apis mellifera* bees from the nectar of flowers or secretions from living parts of plants, and insect-secreted products (Majewska et al. 2019). Among few countries over the world, in Lebanon bees feed on natural sources of nectar all year round, providing for a vast range of honey varieties (Lana and Marwan 2016). Figures from the Lebanese Ministry of Agriculture indicate that there are 6.340 beekeepers in Lebanon own approximately 274.390 beehives (Lana and Marwan 2016).

Honey sold as such must not contain any food ingredient, including food additives, or any other substance that is not part of honey. Bees are sensitive to microorganisms and the main bee diseases are American foulbrood, European foulbrood, and Varroosis (Vidal-Naquet 2012). The treatment of bees requires the use of antibiotics, for this the risk of the presence of residues in the honey is not negligible (Johnson and Jadon 2010).

Residues of antibiotics, such as tetracycline (TC), florfenicol (FF), and penicillin G (PG), in foods of animal origin such as honey, as well as their presence in the environment are of increasing interest because low levels of antibiotics can promote proliferation of bacterial resistance to antibiotics (Serwecińska 2020). In honey, their presence causes adverse effects on humans such as allergic reactions, toxic effects, and damage to the central nervous system. In terms of food and human safety, antibiotics are not authorized for the treatment of bees in the European Union (EU) (Lima et al. 2020; Bonerba et al. 2021) as well as in Lebanon, thus no MRLs established for antibiotics in honey (Forsgren 2010). However, in the absence of monitoring in this sector, there is a reasonable possibility to find contaminated honey in the Lebanese markets.

Some countries have established maximum residue limits (MRL) for TCs in honey, for example, 10 µg/kg in Russia and 50 µg/kg in Britain. In contrast, neither Codex Alimentarius nor the European Union (EU) has developed MRLs for veterinary medications in honey, and the use of antimicrobials in beekeeping is illegal in EU member countries (Commıssıon Regulatıon 37/2010 of 22 December, 2009) (European Commission 2010).

The control of antibiotic residues in foodstuffs of animal origin is carried out in two stages the search for an antibiotic effect by a screening method (microbiological, immunological, or physicochemical) and the confirmation of the presence of the antibiotic by a physicochemical method (liquid chromatography coupled with UV detection, fluorimetry or mass spectrometry) (Gaudin 2016).
Screening and detection methods are qualitative methods designed to distinguish positive samples from negative samples. The screening methods must be supplemented by confirmatory methods which are applied to the samples detected positive by the screening methods.
The chromatographic methods and electrophoresis produce precise results on the level of antibiotic residues (Orso et al. 2016; Dawadi et al. 2021).
HPLC is a physicochemical method that allows the detection and quantification of residues of a fairly wide range of antibiotics extending to all families used in human and veterinary medicine.
This is a much more selective and sensitive method than microbiological methods because it allows molecules to be identified separately and therefore avoids possible interference problems between substances (Bensakhria 2016; Peris-Vicente et al. 2022). Thus reversed-phase HPLC (RP HPLC) has become the most common method of separation and analysis (Shabir 2010).

To the best of our knowledge, there is no single HPLC method reported for the simultaneous determination of the selected three antibiotic compounds of three different therapeutic classes including β-lactams, tetracyclines, and amphenicols in honey samples. This work aimed to develop and validate a method for simultaneous determination of TC, FF, and PG residues in honey using HPLC with DAD and a simple sample preparation technique.

2. Materials and Method

2.1. Chemical and Reagents

HPLC gradient grade acetonitrile (ACN) and methanol (MeOH) were purchased from VWR chemicals. Oxalic acid, and potassium dihydrogen orthophosphate anhydrous (KH_2PO_4) were obtained from Analar. Citric acid anhydrous was purchased from HIMEDIA Laboratories. Disodium ethylenediamine tetra acetate (EDTA), Penicillin G potassium salt, and Formic acid (FA) were purchased from Sigma-Aldrich. Tetracycline, and Florfenicol was generously provided by Pharmadex S.a.l. (medicine factory, Beirut, Lebanon). All aqueous solutions were prepared with ultra-pure water (TKA, Micromed, Germany). The solid-phase extraction procedures were carried out using Waters SupelTM-Select HLB cartridge (200 mg, 6 mL) provided by Sigma-Aldrich.

2.2. Apparatus

All chromatographic readings were done using an HP 1100 Series LC system (Hewlett Packard, Palo Alto, CA, USA) equipped with a quaternary pump, a vacuum degasser, a column compartment, an auto sampler, and a diode-array detector, and controlled by the HP Chemstation chromatography software. For the method that will be adopted, the analytical column was Zorbax Eclipse XDB C8, (5 µm, 150 x 4.6 mm) (from Hewlett Packard, Palo Alto, CA, USA). Other equipments such as pH meter CG 820 (SCHOTT GERATE, made in West Germany), electronic weighing balance (RADWAG Wagi Electronic, Poland), Spectrafuge 6C compact centrifuge (Edtexison, NJ USA), Ultrasonic cleaner (BRANSON 200, made in Taiwan) and vortex made by Daihan Scientific Co. (Korea) are also used in this study.

2.3. Preparation of Standard Solutions

In order to obtain a final concentration of $1mg.mL^{-1}$, a stock standard solution of FF, TC, and penicillin was prepared by dissolving 1 mg of the compound in 1 mL of ACN, MeOH, water/ACN (*v/v*; 1/1) respectively. The solutions were stored in dark vials at + 4 °C until further use. Working solutions were prepared daily by appropriate dilution of aliquots of the standard stock solutions in ultra-pure water. The working solutions were used for sample spiking for the preparation of calibration curves of 6 different concentrations.

2.4. Chromatographic conditions

The elution was conducted using a mobile phase system consisting of a mixture of KH_2PO_4/ACN (80:20). The mobile phase was mixed and sonicated for 5 min and then vacuum filtered through a 0.45 µm nylon filter.
Chromatographic separation of the analytes was achieved on Zorbax Eclipse C18 column, under isocratic mode allowing complete analysis in less than fifteen minutes. The flow rate was adjusted at 1 mL/min and the column thermostat was set at 35 °C. The injection volume was 25 µL, and the final run time of the method was 15 min. Detection wavelengths were set at 224 nm for FF, 230 nm for PE, and 350 nm for TC. While data analysis was performed utilizing the Hewlett-Packard ChemStation software.

2.5. Extraction and clean-up procedure

Extraction and clean–up procedures for samples were performed following the protocol of Kumar et al. (2020) with slight modifications. An aliquot of the honey sample (2.5 g) was taken in a 50 mL centrifuge tube. Then samples were dissolved in10 mL of 0.1M EDTA-McIlvaine buffer (pH 4.0) (prepared as described by Cinquina et al. (2003) followed by vigorous shaking for 5 min. The sample was then centrifuged at 6000 rpm for 10 min. The supernatant was collected and passed through a disposable Whatman membrane filter 0.45 µm (Whatman, Maidstone, UK) to remove any remaining milk flakes. Clean up of the extract was done by using SPE method. The filtrate was loaded on a Supel Select HLB (Hydrophilic-Lipophilic Balance) cartridge preconditioned with 3 mL of methanol followed by 2 mL of ultra-pure water under pressure. The antibiotics were eluted with 1.5 mL of MeOH after the sample cartridge was rinsed with 2 mL of water. The elute was collected and filtered through a 0.45 µm syringe filter before being kept in vials for further analysis.

2.6. Method validation

Before the validation steps, an optimization step was conducted, chromatographic parameters, including composition and flow rate of the mobile phase, gradient elution, injection volume, and column temperature, were studied in order to find the optimum conditions for chromatographic separation of all chemicals in a short amount of time.
The characteristics and the procedures used for validation were performed following the recommendations from the Commission Decision 2002/657/EC of the EU (2002), for the parameters selectivity, linearity, recovery (accuracy), decision limit (CCα), detection capacity (CCβ), and precision. The LOD and LOQ were calculated according to the guideline of the International Conference of Harmonization (ICH) Guidelines (Abraham, 2010). For the validation studies, the work solution was prepared by spiking the appropriate volume of working FF, PE, and TC standards in a blank honey sample (antibiotic-free).

2.7. Statistical analysis

All analyses were performed using Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA). Mean, standard deviations, range, R^2, % RSD, etc. were calculated for each targeted analyte using descriptive statistics.

3. Results and discussion

3.1. Optimization of chromatographic conditions

To achieve satisfactory chromatographic separation and high sensitivity, different solvent systems, to design suitable mobile phases, and columns were optimized. An aqueous mobile phase consisting of potassium dihydrogenophosphate (0.05 M) was the best system, with ACN and MeOH being examined as organic solvents to increase the sensitivity. Improved ATBs identification using ACN may be linked to MeOH's role in TC degradation. Liang et al. (1998) found that the degradation of TC is increased in MeOH solutions via functional group substitutions or additions to TC. The results of this study agreed with findings from these previous studies. This mobile phase still contains relatively high amounts of salts 80 % KH_2PO_4 to be used in conventional reversed-phase analytical columns. The initial mobile phase tests were conducted using a brand C18 column Hypresil-ODS, but the separation efficiency decreased in a short period of use. Thus, it was decided to use a column that resists mildly acidic conditions (Zorbax Eclipse Plus C18). for better selectivity, resolution, and to maximize the retention of FF and PG we used the Zorbax Eclipse which displays a good analysis and the peaks are well distinguished and to increase the sensitivity of the column. The mobile phase was tested to evaluate the separation and responses (analyte area) of a 200 µL of stock solution for each antibiotic, fortified blank honey sample with TC, FF, PG, and observations of peaks according to wavelengths respectively 350 nm, 224 nm, 230 nm.

Using the ODS Hypersil C18 column results showed that reducing the modifier component (MeOH and /or ACN) of the mobile phase decreased the retention times of ATBs involved in this study and generally deteriorates the separation among all of them (Table 1).

Table 1. Effect of mobile phase and column on analytes retention times.

%(KH_2PO_4/ ACN/MeOH)	Rts (min)			Column
	TC	PG	FF	
(90 /10/0)	2.8	3.3	9.9	ODS Hypersil C18
(90 /0/10)	2.8	2.8	13.4	ODS Hypersil C18
(80 /20/0)	2.3	3.1	3.6	ODS Hypersil C18
(75 /25/0)	2.6	3.2	3.5	ODS Hypersil C18
(40/40/20)	1.2	1.3	1.2	ODS Hypersil C18
(80/20/0)	3.2	6.6	9.7	Zorbax Eclipse. Plus

3.2. Method validation

The linearity response was examined by the external standard method. For this purpose, triplicate analysis of milk samples fortified with FF, PE, or TC at seven fortification levels ranging from 0.004 to 5 ppm were prepared and injected in triplicates. The standard calibration curves were generated for each analyte by plotting concentrations against the peak area of the analyte. The validating parameters of each calibration curve (slope (a), intercept (b), and correlation coefficient (R^2) are shown in Table 2. The correlation coefficient ranging between 0.9954 and 0.9969, indicates a strong linear relationship between the concentration of the analyte and the area under the peak.

The sensitivity of the method, i.e. the change in response on a measuring instrument divided by the corresponding change in stimulus, was represented by the slope of the calibration curve (Prichard et al. 2001).

The specificity of the method is defined as the ability to distinguish between an analyte and other substances (United Nations Office on Drugs and Crime & Laboratory and Scientific Section 2009). It was investigated by analysis of ten different blank milk samples to determine any interfering peaks from endogenous compounds.
LOD and LOQ established for this method were calculated from the standard deviation (σ) of y-intercepts of regression analysis and the calibration curve slope (m), according to equations 1 and 2 respectively (Abraham 2010).

$$LOD = 3.3\frac{\sigma}{m} \quad \text{(eq. 1)}$$

$$LOQ = 10\frac{\sigma}{m} \quad \text{(eq. 2)}$$

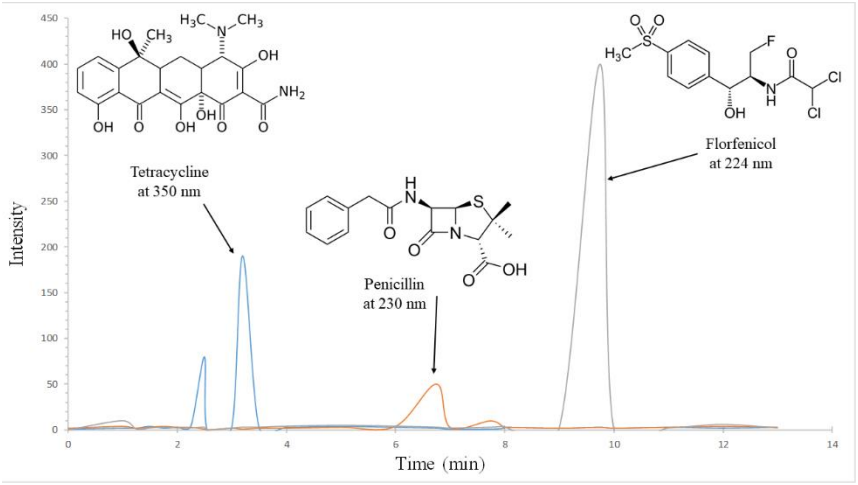

Fig. 1. Chromatograms of fortified honey extract.

Table 2. Regression analysis, LOD, and LOQ of TC, FF, and PG.

Antibiotic	Range ($mg.kg^{-1}$)	Slope	Intercept	Correlation coefficient	LOD ($mg.kg^{-1}$)	LOQ ($mg.kg^{-1}$)
TC	0.7 -17.5	0.0007	-0.2584	0.9969	0.514	1.85
FF	0.7 -17.5	0.01608	-1.5479	0.9963	0.571	1.90
PE	0.7 -17.5	0.0005	0.6675	0.9954	0.606	2.02

The decision limit (CCα) and the detection capability (CCβ) in the case of substances for which no authorized limit has been set, can be determined using the matrix-matched calibration curves. CCα was determined as the "corresponding concentration at the y-intercept plus 2.33 × the standard deviation of RSDR", while CCβ was calculated as the "concentration at the decision limit plus 1.64 × the standard deviation of RSDR" (European Commission 2002). The CCα values ranged from 588 to 863 $\mu g.kg^{-1}$, whereas the CCβ values varied in a range from 457 to 1000 $\mu g.kg^{-1}$. (CCβ) is above the limit of detection in all three antibiotics (

Table **3**).

Table 3. Results for decision limits (CCα), and detection capabilities (CCβ) obtained for the analyzed ATBs in Honey.

ATB	$mg.kg^{-1}$	
	CCα	**CCβ**
TC	0.710	0.609
FF	0.588	0.457
PG	0.863	1

The precision of the method consists of intra-assay precision and inter-assay precision, which was checking the percentage of relative standard deviation (% RSD) of peak areas. The intra-assay precision was confirmed by enriching honey blank sample with antibiotics of interest TC, PG, FF, at a single concentration level respectively (0.02 $mg.mL^{-1}$; 0.015 $mg.mL^{-1}$; 0.0125 $mg.mL^{-1}$) for 3 days (interday), and 11 injections per day (intraday). The data of the repeated analysis are shown in Table 4.

Table 4. Precision test of the method

AC	SD	RSD (%)	Intraday CV (%)	Interday CV (%)
TC	0.324	3.59	2.22	2.53
FF	3.71	4.42	2.23	3.06
PG	0.653	9.87	10.02	11.12

The CV for intraday precision varied from 2.22 % to 10.02 % and the CV for interday precision varied from 2.53 % to 11.12 %. These results are in agreement with the requirements set by the decision 2002/657/EC from the European Union, which is 10 to 20% depending on the concentration of the analyte. % RSD values for peak areas indicate the high precision of the chromatographic system.

3.3. Lebanese honey sample results:

In the absence of any study, to the best of our knowledge, dealing with the residues of antibiotics in honey in Lebanon, we have tried to apply the method to a small sample of honey. Ten honey samples were analyzed. Three of them are gratefully provided by three different farms from the south of Lebanon, the remaining are bought from the Lebanese market. Obtained results show that the samples contain no trace of these three antibiotics at the LODs of the method since chromatograms do not show any peaks on the specific retention times.

It is difficult to refer to Lebanese similar studies in order to compare the numbers we obtained or simply refer to them.

This research paper lays the first building block for a more comprehensive study that will cover all Lebanese regions at a later time, especially in the absence of monitoring and awareness of beekeepers, who are often not subject to any monitoring by the concerned national institutions.

4. Conclusion

To the best of our knowledge, this is the first study in which a fast and reliable method has been developed and validated for simultaneous detection and quantification of FF, PE, and TC in honey. The developed method validated according to recommended criteria of Commission Decision (EC) No 2002/657/EC provided good performance and satisfactory recovery, thus results showed the applicability for routine analysis of honey. Then, the validated method served to detect and quantify FF, PE, and TC residues in two samples from Lebanese farms. The overall results showed the absence of these antibiotics residues in the collected samples. Finally, this validated method can be applied to conduct a comprehensive survey of the whole Lebanese territory by analyzing a sufficient number of samples.

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Authors' contributions:

The article is written and designed by A.J., E.C.; Data analyzes were determined by A.J.; Experiments was done by B.R.

Conflict of interest disclosure:

The authors declare that there is no real, potential, or perceived conflict of interest for this article.

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