METHOD DEVELOPMENT FOR CIPROFLOXACIN ANALYSIS IN HONEY BY LC-MS/MS

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Abstract. *Ciprofloxacin is a veterinary drugs authorized for use in food animal production. The analysis of residual amounts of drugs in food from animal origin is important for quality control of products for consumers. Due to its importance, a method was developed for screening and confirming ciprofloxacin residue in honey samples. Method development consisted in preparing the standard solutions, optimizing the compound and source dependent parameters, choosing the mobile phase and the appropriate HPLC column followed by processing a calibration curve and finally injecting a real sample. The honey sample was collected from Transylvania region. Its extraction consisted in acidification and dilution of the sample, extraction on SPE columns, elution with ammonium hydroxide in methanol, evaporation to dry and reconstitution with mobile phase. The extracted sample was then injected on a Phenyl-Hexyl column and detected through a LC/MS/MS system in an ESI (electrospray ionization) mode and the result was considered to be satisfying.*

Keywords: ciprofloxacin, honey, solid phase extraction (SPE), electrospray ionization.

INTRODUCTION

Accurate monitoring of chemical residue levels in food and agriculture products is essential to assure the safety of the food supply. The analysis of chemical residues requires techniques sensitive enough to detect and quantify contaminants at or below the maximum residue limit (MRL) of the compound in a given sample matrix. Maximum Residue Limits have been set up by a European Union Council Regulation on Veterinary Drug Residues [1].

Ciprofloxacin is a synthetic chemotherapeutic antibiotic of the fluoroquinolone drug class [2] (figure 1). It is a second generation fluoroquinolone antibacterial. It kills bacteria by interfering with the enzymes that cause DNA to rewind after being copied, which stops DNA and protein synthesis [3]. Ciprofloxacin is 1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(1-piperazinyl)-3-uinolinecarboxylic acid. Its empirical formula is $C_{17}H_{18}FN_3O_3$ and its molecular weight is 331.4 g/mol. It is a faintly yellowish to light yellow crystalline substance [4].

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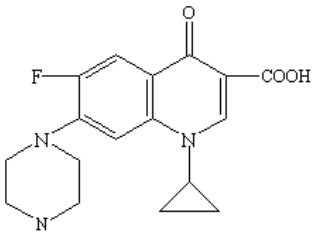

Fig.1. Chemical structure of ciprofloxacin

Experimental

Reagents and standards: The method developed in this study was accomplished using Ciprofloxacin analytical standard (≥98.0%), Methanol LC-MS Optigrade (≥99.8%), Acetonitrile LC-MS Optigrade (≥99.8%) and formic acid ULC-MS Optigrade (≥99%) acquired from LGC Standards. The ultra-pure water was obtained with a Milli-Q water purification system from Millipore. The sample extraction was performed using Strata-X (33µm, 100mg, 12mL) solid phase extraction (SPE) cartridges obtained from Phenomenex.

Standard Solutions Preparation; Stock Solutions (~ 1mg/mL) was prepared dissolving 5 mg of Ciprofloxacin in 5 mL of MeOH using a vortex mixer. In order to optimize the compound dependent parameters (infusion) of the source a solution 10 µL of stock solution were diluted in 100 mL MeOH. The source dependent parameters (FIA-flow injection analysis) were optimize using a solution made from 10 µL of stock solution diluted in A/B 50:50 v/v, where A represents 0.1% FA in water and B 0.1% FA in ACN.

Sample extraction: The cartridge was conditioned with 5mL methanol, followed by 5mL of deionized water. The honey sample (1 gram) is acidified with 1mL of 2M hydrochloric acid, sonicated for 30 min and then treated with 0.3M citric acid in water so as to make up the total volume to 5mL. The ciprofloxacin solution where spiked into this diluted honey solution and loaded onto the cartridge. Washing was done with 5mL of water (in two aliquots), then with 5mL of 50:50 methanol/acetonitrile (in two aliquots) and then the cartridge was dried. Elution was carried out with 3mL of 2% ammonium hydroxide in methanol. The eluate was evaporated to dry. The residue was reconstituted into 1000µL of mobile phase.

Liquid Chromatography conditions: An HPLC system Agilent 1200 Series, consisting in an autosampler Agilent G1316A, column oven G1316A and a binary pump G1312A, was used. The liquid chromatographic column was a Phenomenex Luna Phenyl-Hexyl (C6) 100A, 3µm, 50×3.0 mm. The cromatography was performed by isocratic reverse phase separation with a 40µL injection volume, using a mobile phase of 85% A/15% B at a flow rate of 0.2 mL/min. The column oven was set to 40ºC.

Mass Spectrometry detection: The mass spectrometry detection was carried out with an API 3200 QTRAP LC/MS/MS system using a TurboV in an ESI (electrospray ionization) mode. Multiple reaction monitoring (MRM) transitions were followed to quantify the deprotonated precursor molecular ions $[M+H]^+$ and the related product ion.

Results and Discussion: As the international food trade continues to grow, so does the need for careful monitoring of the food supply to ensure that levels of drug residues and other chemical contaminants are below established standards.

For LC/MS/MS method development several steps must be fulfill. These steps are: choosing the TurboV source ionization (ESI or APCI); choosing the ionization mode (positive or negative); Q1 and Q3 scanning; establishing the compound dependent parameters by infusion; establishing the mobile phase; establishing the source dependent parameters by performing FIA; selecting the column which determinate the flow and the analysis time; performing a calibration curve and injecting real samples.

In order to choose the appropriate method to determine the ciprofloxacin from real samples the study started with the selection of TurboV source ionization. APCI and ESI were evaluated both in positive and negative modes and ESI positive ion mode was found to be the most effective for this compound.

Knowing the molecular mass of ciprofloxacin (331.346), a Q1 positive scanning using the infusion solution shows its presence at 332.2 m/z ratio (figure 2). The compound obtained in Q1 was fragmented in Q2 and the product ion was obtained in Q3 at 231.3 (figure 3).

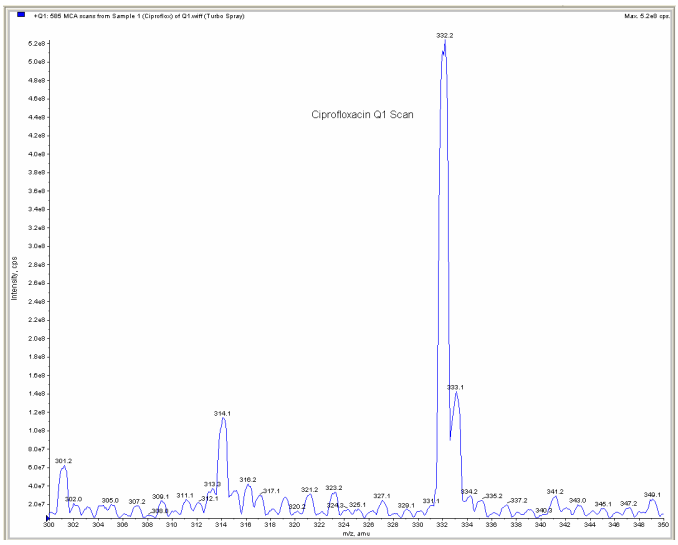

Fig. 2. Chromatogram obtained for 100 ng/mL ciprofloxacin in infusion solution by Q1 positive scanning.

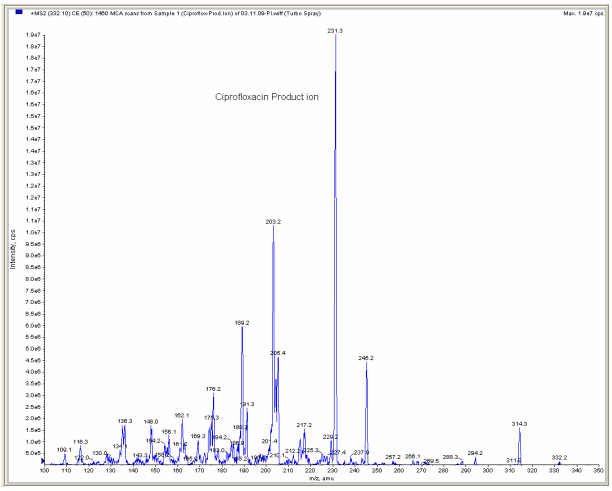

Fig. 3. Chromatogram obtained for 100 ng/mL ciprofloxacin in infusion solution by Q3 positive scanning.

The next step was to optimize the compound dependent parameters of the source using the infusion solution with an injection flow of 10µL. The following parameters were obtained:

DP (Declustering potential): 46.0V;

EP (Entrance potential): 7V;

CEP (Collision cell entrance potential): 18.0V;

CXP (Collision cell exit potential): 4.0V.

The precision of the obtained parameters was check by using ramp parameter settings, individually, for every one of them. The results proved their accuracy.

As a result of scanning and optimizing the compound dependent parameters, a MRM method (Multiple Reaction Monitoring) was developed as a further step to develop a LC/MS/MS method. This method includes also the LC system with the parameters presented earlier. The response of the system to the new MRM method using a solution of 100 ng/ml in A/B 50:50 v/v was presented in figure 4.

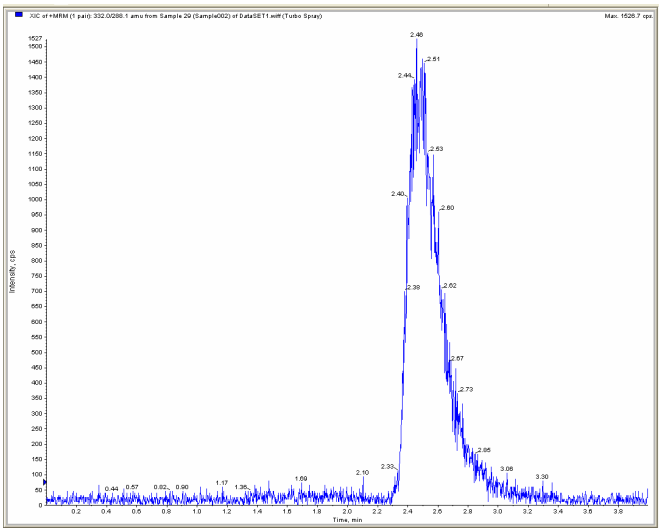

Fig.4. Chromatogram obtained for 100 ng/mL ciprofloxacin in FIA solution by MRM method following the 332.2→231.3 transition.

In order to obtain the appropriate mobile phase, using literature studies,[5],[6],[7],[8] different proportion of A and B were studied starting with A/B 50:50 v/v up to 100% A and then up to 100% B. Two injections were performed for every combination of A and B with a 0.2 mL/min flow in a 20 min scanning time. The best mobile phase was indentified as A/B 85:15 v/v.

Analyst program allows the automatic MS/MS optimization of the source dependent parameters using the selected mobile phase, FIA solution and a restriction column. After FIA analysis the following values of the source dependent parameters were obtained:

CUR (Curtain Gas): 38 psi;
IS (IonSpray Voltage): 5500V;
TEM (Teperature): 550°C;
GS1 (Nebulizer Gas): 40 psi;
GS2 (Turbo Gas): 35 psi:

The next step consisted in HPLC column selection. From 3 available columns, Phenomenex Luna C18 3µm, 50×2.0 mm, Phenomenex Gemini 3µm, 50×3.0 mm and Phenomenex Luna Phenyl-Hexyl 3µm, 50×3.0 mm, the last one proved to be more apropriate in the scanning conditions mentioned above, due to the shape and reproductibility of the chromatographic peak. The column was washed after every 15 injection for 30 min at 0.2 mL/min flow with Water/ACN 50:50 v/v in order to remove the formic acid.

In order to verify the developed method a calibration curve was made in the range of 1 – 75 ng/mL. A calibration curve with a correlation factor of r=0.9954 was

obtain using a linear regression function (1/x·x). The calibration curve is presented in figure 5 and the data results are presented in table 1.

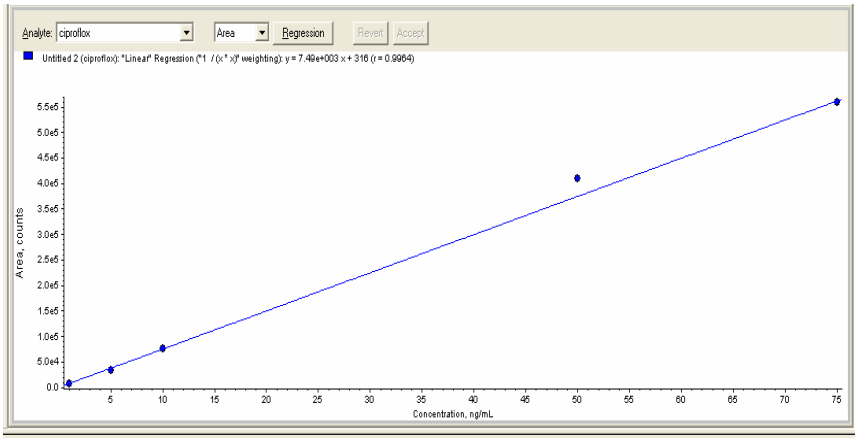

Fig.5. Ciprofloxacin calibration curve following 332.2→231.3 transition.

Tabel 1

Data results for ciprofloxacin calibration curve following 332.2→231.3 transition.

Sample Name	Analyte Peak Area (counts)	Analyte Peak Height (cps)	Analyte Concentration (ng/mL)	Calculated Concentration (ng/mL)	Accuracy (%)
1	7.96e+003	4.57e+002	1.00	1.02	102
2	3.32e+004	1.76e+003	5.00	4.40	87.9
3	7.61e+004	3.64e+003	10.0	10.1	101
4	4.10e+005	2.09e+004	50.0	54.7	109
5	5.58e+005	5.58e+004	75.0	74.5	99.4

One honey sample collected from Transylvania region was extracted using the sample preparation method mentioned above and studied with the developed method. The results showed the presence of the ciprofloxacin and its area was well fitted in the calibration curve and its concentration was determinate with the developed method.

During method development, the influence of the environmental conditions on the stability of the solutions was observed. It was concluded that no special environmental conditions for sample preparation were necessary.

CONCLUSIONS

A rapid, sensitive and reliable method for quantitation of 17β-estradiol in waste water was developed using a 3200 QTRAP LC/MS/MS system with a TurboV in an ESI negative (electrospray ionization) mode. Specific compound and source dependent parameters were developed for MS instrument. Parameters for LC instrument were also established. A special interest was given to the flow rate, mobile phase and column selection.

A calibration curve was selected based on bibliographic studies and the method has been proved to have satisfying results on real honey samples.

This method will be extended in future in order to obtain even lower detection limit and a better recovery. Also, its extinction for simultaneous analysis of multiple antibiotics from honey is expected.

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