Development of a new UFHPLC-MS method for determination of different antibiotics in animal feeds

Development of a new UFHPLC-MS method for determination of different antibiotics in animal feeds

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Overview

- In Europe, antibiotics could be add into animal feeds exclusively for therapeutic use;
- Antibiotics in animal feeds enhance human resistance against bacterial infections;
- A rapid and sensitive UFHPLC-MS/MS method to determine different antibiotics classes into animal feed was developed;
- The developed method was applied to bovine meat samples.

Introduction

Since from the middle of the last Century, antibiotics were added at sub-therapeutic doses in animal feed as animal growth promoters.

The possible action mechanism involves the suppression by antibiotics of huge intestinal bacterial populations, which consume energy during fermentation process. So, this remaining energy could be re-used for animal growth.

As consequence, the meat has less fat proportion, enhanced muscle mass and high protein percentage [1].

However, the antibiotics abuse leads to the development of bacterial resistance. Consequently, many infections result incurable [2, 3].

Therefore, from 2006 antibiotics as growth promoters are banned in Europe [4] and they can only be used as therapeutic drugs when infection occurs. Animals under antibiotic treatment could not be slaughtered before an average of 28 days [5, 6]. If this time is not respected, there is a potential risk of finding antibiotics traces in meat.

A new rapid and sensitive UFHPLS-MS/MS method to detect traces of different classes of antibiotics (sulfonamides, ß-lactams, lincosamides, pleuromutilins, macrolides and diaminopyridine) in animal feeds and meats was developed. A validation procedure was carried out with definition of LOD, LOQ, precision and repeatability and reproducibility.

Materials and Method

Sample Preparation

- Animal Feeds Solid-Liquid Extraction:
  - animal feed samples spiked with different amounts of antibiotics mix solution were extracted under stirring with methanol, filtrated and analyzed.
- Bovine Meat SPE Extraction:
  - meat samples spiked with different amounts of antibiotics mix solution were flushed with acetone/dichloromethane /acetic acid 50:50:10;
  - SPE column (Waters Oasis HLB 60 mg and Agilent Technologies Bond Elute 500 mg) were used as follow: equilibrate with hexane, then acetone/dichloromethane/acetic acid 50:50:10, load the samples, wash with methanol, elute with methanol/ammonium hydroxide 2:1;
  - dry the samples under stream of N_2 and re-dissolve in ammonium acetate 5 mM pH 3/Methanol 8:2.

NEXERA LC-30AD (Shimadzu) Conditions

- Kinetex 2.6 µm C18 100Å 100 × 2.1 mm (Phenomenex);
- Eluents: Acetonitrile (A) and Formic Acid 0.05% (B);
- Gradient Condition: from 10% to 50% of solvent A in 25 minutes;
- Injection volume: 10 µL; Flow rate: 500 µL/min

QTRAP5500 (Turbo Ion Spray, ABSciex) Settings

- MRM are listed in Table 1

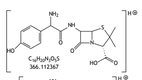
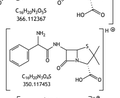
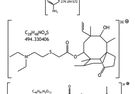
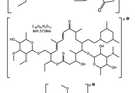
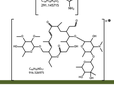
Analyte	Structural Formula	Q1 (m/z)	Q3 (m/z)	Time (sec)	DP	EP	CE	CXP
Amoxicillin		366	349 @ 207	30	100	5	10 @ 15	18 @ 11
Ampicillin		350	106 @ 192	30	170	10	20 @ 21	15 @ 12
Lincomycin		407	359 @ 126	50	210	8	24.7 @ 34	23 @ 17
Sulfadiazine		251	156 @ 92	30	70	4	19.7 @ 33	1.5 @ 1.3
Sulfadimethoxine		311	156 @ 108	30	98	6	27 @ 34.6	11 @ 19
Sulfamerazine		265	156 @ 172	50	60	5.7	21.3 @ 21.4	9 @ 19.8
Sulfamethazine		279	186 @ 124	50	80	5.5	24.1 @ 29.6	12 @ 8.7
Tiamulin		494	192 @ 119	30	220	10	27 @ 57	16 @ 13
Tilmicosin		435.1	522 @ 696.5	50	120	10	24	25 @ 30
Trimethoprim		291.3	230 @ 123	30	16.7	10	35.5 @ 54.7	50 @ 14
Tylosin		916.6	174 @ 772.5	50	60	5	47 @ 39	10 @ 34

Table 1: Multiple Reactions Monitor for analyzed antibiotics.

Results and Discussion

The chromatographic run gave a good separation between the different analytes, with only one co-elution (ampicillin together with sulfamethazine, Rt= 10.8 min). Figure 1 shows a chromatogram of pure standard in aqueous solution.

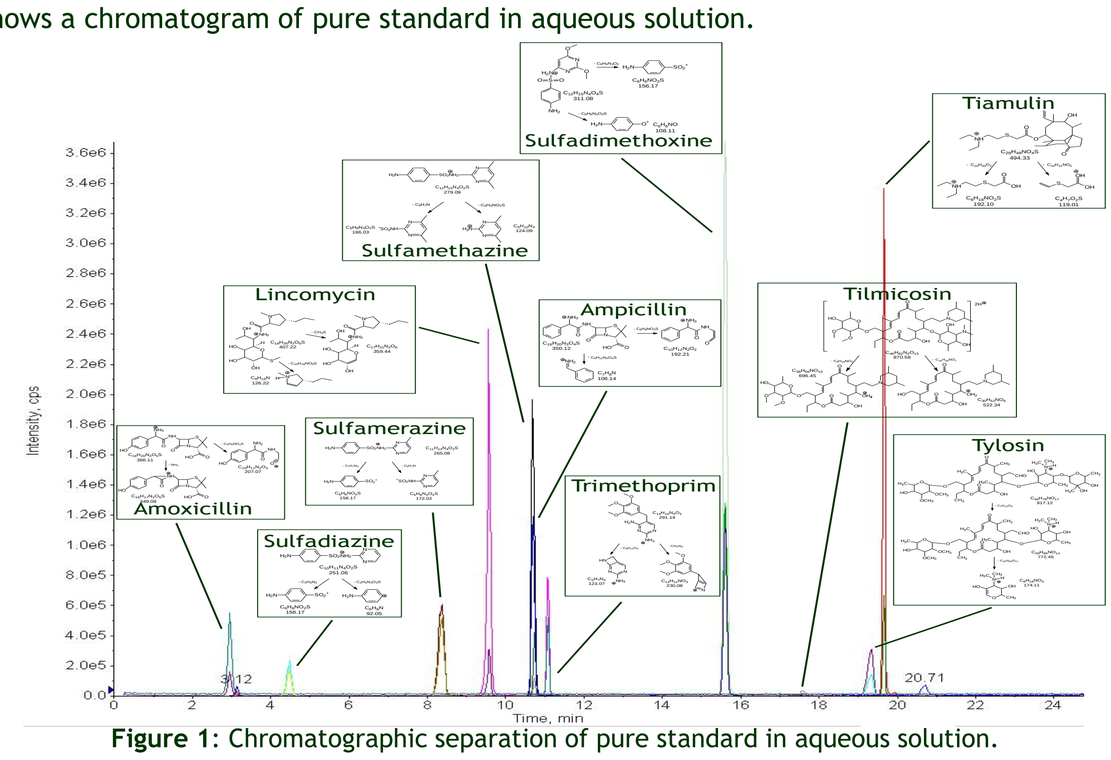


Figure 1: Chromatographic separation of pure standard in aqueous solution.

The method was validated in terms of linearity, relative standard deviation of inter and intra day precision with pure standards both in water solution and in animal feed after solid-liquid extraction. Table 2 and Figure 2 illustrate the results in water and in animal feeds respectively.

In animal feeds, the limit of quantitation was evaluated, and the values are reported in table 3. Figure 4 illustrates the yields of solid-liquid extraction of antibiotics.

Moreover, the developed method was tested on muscles meat samples from bovine and the yields of two different SPE (Bond Elute, Agilent Technologies; HLB, Oasis Waters) was evaluated (Figure 4).

Analyte	R^2	RSD% inter day	RSD% intra day	LOD (ng/Kg)	LOQ (ng/Kg)
Amoxicillin	0.9920	31.6	22.4	100	500
Ampicillin	0.9798	23.1	9.0	21	50
Lincomycin	0.9866	13.2	15.6	26	50
Sulfadiazine	0.9849	14.0	22.2	6	50
Sulfadimethoxine	0.9794	11.2	17.1	17	50
Sulfamerazine	0.9872	9.2	11.9	14	50
Sulfamethazine	0.9895	8.4	15.2	6	50
Tiamulin	0.9751	16.0	22.9	5	50
Tilmicosin	0.9574	26.2	33.0	200	500
Trimethoprim	0.9851	10.2	11.9	9	50
Tylosin	0.9851	23.2	20.1	81	500

Table 2: Linearity (R^2), relative standard deviation (RDS%) of inter and intra day of pure standards in water solution.

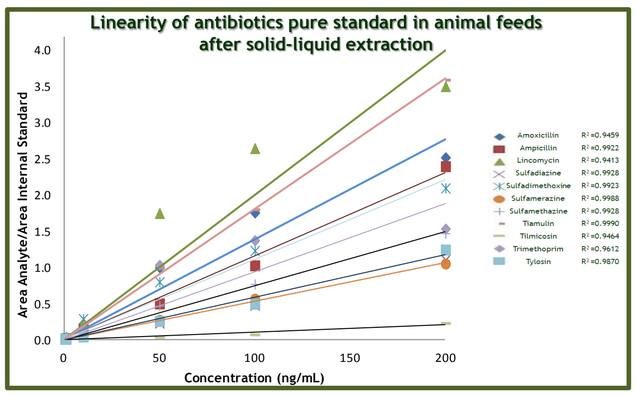


Figure 2: Linearity (R^2) of pure standards in animal feeds matrices after solid-liquid extraction.

Analyte	Experimental LOQ (µg/Kg)
Amoxicillin	1070 ±22.1
Ampicillin	59 ± 12.6
Lincomycin	55 ± 13.2
Sulfadiazine	50 ± 16.3
Sulfadimethoxine	52 ± 0.8
Sulfamerazine	54 ± 22.6
Sulfamethazine	51 ± 15.6
Tiamulin	62 ± 10.3
Tilmicosin	64 ± 5.3
Trimethoprim	57 ± 5.8
Tylosin	63 ± 29.1

Table 3: List of experimental LOQ of analyzed antibiotics in animal feeds matrices.

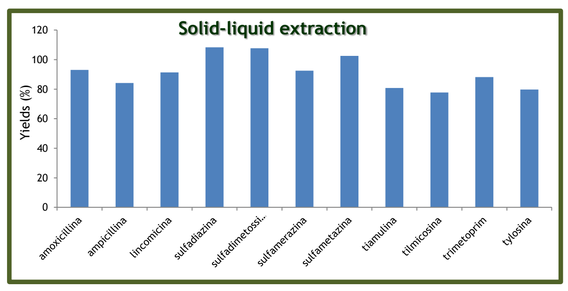


Figure 3: Percentage yields of solid-liquid extraction in animal feeds samples.

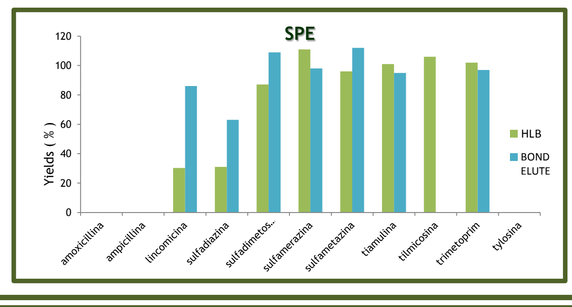


Figure 4: Percentage yields of SPE extraction in bovine meats samples.

Conclusions

- The developed UFHPLC-MS/MS method was fruitfully used to qualitatively and quantitatively determine different classes of antibiotics both in animal feeds and bovine meats;
- Solid-liquid extraction protocol for animal feeds and HLB-SPE protocol for bovine meats give good yields, up to 82% and 38% respectively;
- The chromatographic separations of eleven antibiotics is obtained in only 25 minute long run;
- The sensitivity is very high due to the QTRAP5500 intrinsic performance, but especially due to the optimization of MS parameters, such as source gases and temperature, potentials, collision energies and so on.
- The precision, repeatability and reproducibility of the developed method were evaluated and the results are excellent.

References

[1] Jensen BB. 1998. J Anim Feed Sci 7: 45-46.
[2] WHO. 2012. "The evolving threat of antimicrobial resistance: options for action".
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[5] Gazzetta Ufficiale Italiana N. 78 del 3 aprile 1993, S.O., Decreto Legislativo 3 marzo 1993, N.92.
[6] Gazzetta Ufficiale Italiana N. 113 del 17 maggio 2005, Decreto Legislativo 3 marzo 2005.