Effect-directed analysis of antibiotics in milk

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Introduction

Antibiotics are widely used for therapeutic and prophylactic purposes in human and veterinary medicine. In regard to dairy animals, to avoid risks related to drug residues in milk, maximum residue limits (MRL) have been established in many countries for each antimicrobial agent. In the European Union, the MRLs (EU-MRL) in foodstuffs of animal origin are established by the *Codex Alimentarius* Commission [1], the Regulation (EC) n. 470/09 [2], repealing the Council Regulation n. 2377/90 [3], and the Commission Regulation (EU) n. 37/10 [4]. Reduction of the level of antibiotic contamination in milk is important for two reasons, both the potential risk to human health (allergy, bacterial resistance) and the effect on milk manufacturing processes. The sources of contamination are 61 % of residue test failures to lactation intra-mammary, 31 % to dry cow intra-mammary, 6 % to injections and 1 % to other causes [5]. In the following, a non-target effect-directed analysis was performed using planar chromatography in direct combination with a bioassay suited for detection of important antibiotics [6]. Using this direct bioautography method, also non-targeted antibiotics would be revealed.

Results and discussion

HPTLC-FLD-bioassay

Six milk samples with different fat content (M1: lactose-free 1.5 %, M2: fresh 1.5 %, M3: fresh 3.5 %, M4: UHT 0.1 %, M5: UHT 1.5 %, and M6: UHT 3.8 %) were spiked with the fluoroquinolones ciprofloxacin and marbofloxacin. After dilution with acetonitrile, the samples were applied on an HPTLC plate silica gel 60 (Merck). The development was carried out with a solvent mixture of isopropyl acetate – methanol – ammonia, 2:2:1. The dried chromatogram was evaluated (Fig. 1A). After UV/Vis spectra recording, the optimal absorbance measurement wavelengths were found to be 280 and 300 nm for ciprofloxacin and marbofloxacin, respectively. However, matrix interference was evident at these measurement wavelengths, and measurement at 320 or 340 nm was a compromise. Fluorescence measurement at 313/400 nm lead to an increased signal intensity for both antibiotics (Fig. 2). The quality of the calibration (7.5-75 ng/zone) was acceptable (Fig. 3). The evaluated chromatogram was dipped in a *Bacillus subtilis* (ATCC 6633) broth, incubated, and visualized with a tetrazolium salt solution [6] (Fig. 1B).

Fig. 1 Chromatograms of ciprofloxacin (hR_F 51±3) and marbofloxacin (hR_F 79±4) standard mixture (S1 - S4, 7.5 - 75 ng/zone) and six milk samples (M1 - M6) spiked with both fluoroquinolone antibiotics (A) at UV 366 nm and (B) after *Bacillus subtilis* bioassay

Validation

The limits of quantitation [7] of ciprofloxacin and marbofloxacin in milk matrix were below 60 µg/L. Using the bioassay detection, LOD of ciprofloxacin was improved; additionally illegally used antibiotics could be detected.

The precision and accuracy of the method was evaluated according to Council directive 96/23/EC [8]. The achieved precision in matrix over the whole procedure was <7 % (intra-day, n=5) and <10 % (inter-day, n=5). The recoveries (6 different milk matrices spiked at 1 mg/L) ranged 77-85 % for ciprofloxacin and 85-92 % for marbofloxacin with a reproducibility of <8 % and <7 %, respectively.

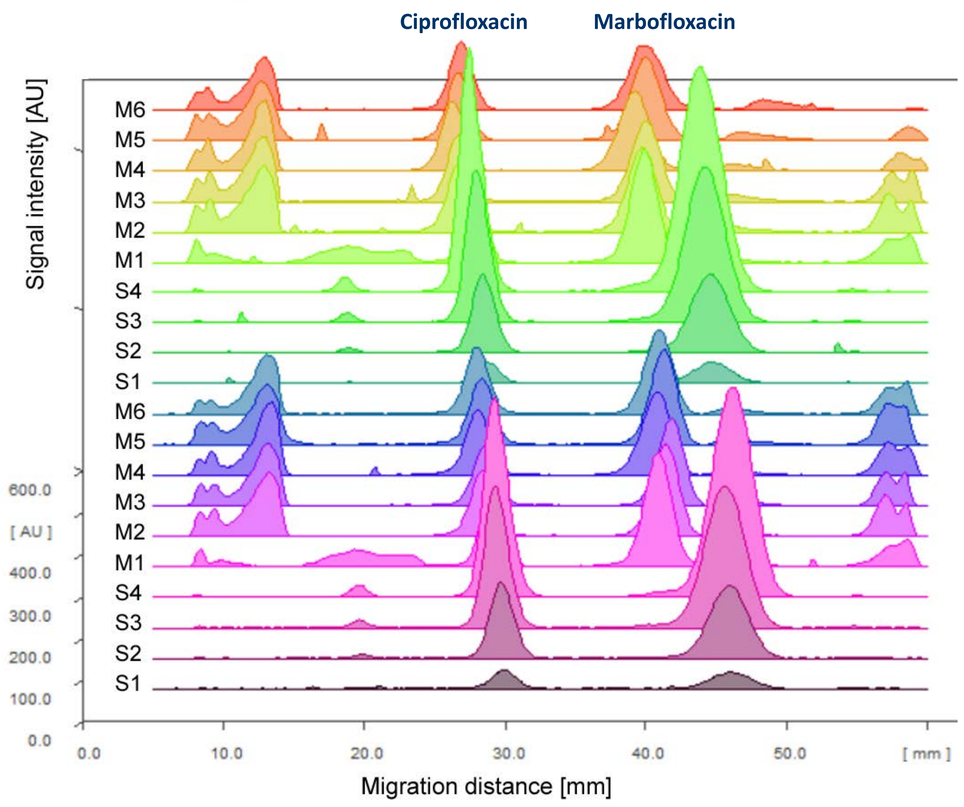

Fig. 2 3D densitogram of fluorescence measurement at 313/400 nm showing standard mixture (S1 - S4, 7.5 - 75 ng/zone) and six milk samples (M1 - M6) spiked with both fluoroquinolone antibiotics

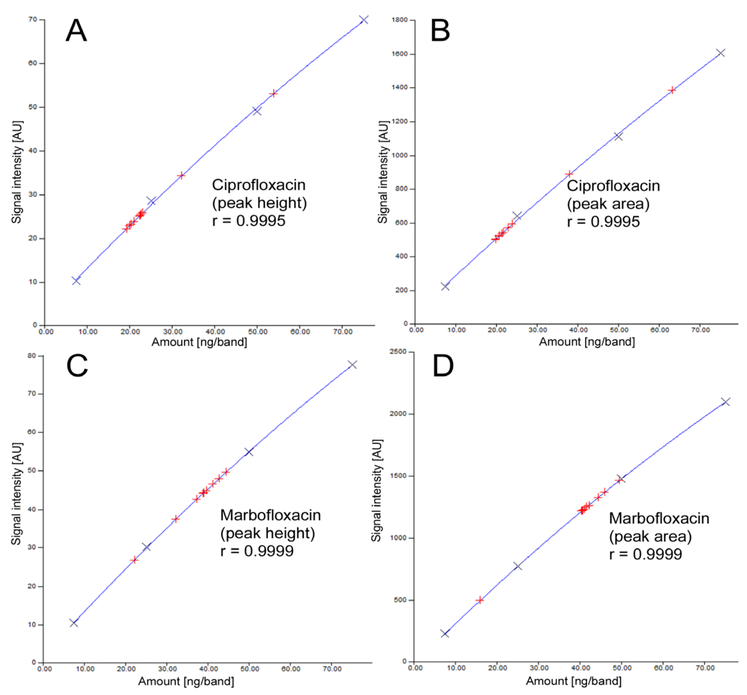

Fig. 3 Polynomial calibrations via peak height and area of (A and B) ciprofloxacin and (C and D) marbofloxacin with good correlation coefficients (r)

Conclusion

The non-target HPTLC-FLD-bioassay method for quantitative determination of two fluoroquinolones in milk is inexpensive and fast, if compared to GC and HPLC analyses. Additional information is obtained through the non-target bioassay detection. For both antibiotics, this method can be used successfully. However, the sensitivity of *Bacillus subtilis* (ATCC 6633) to other group antibiotics has still to be investigated. Further bacteria or different strains of *Bacillus subtilis* will be considered with regard to a reduced detectability of antibiotics .

References

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