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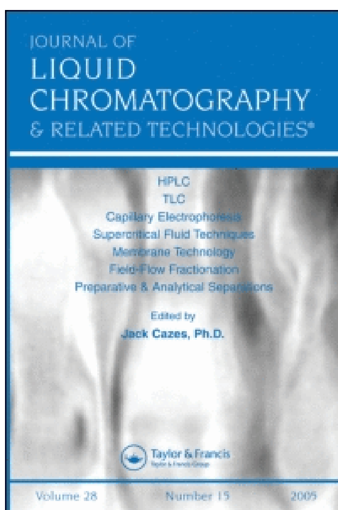

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Thin-Layer Chromatography-Direct Bioautography of Flumequine Residues in Milk

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Abstract: Fluoroquinolone antibiotics are a relatively new group of synthetic antibiotics derived from 3-quinolone carboxylic acid. They are widely used in both human and veterinary medicine. Flumequine is frequently used in fish, poultry, and cattle husbandry for the treatment and prevention of diseases. It is also used to enhance weight gain of livestock as a feed or water additive. Hence, the antibiotic residues may be present in edible products, e.g., in meat, eggs, or milk. Thin-layer chromatography–direct bioautography combines TLC with microbiological detection. In the present paper, conditions for bioautographic detection of flumequine were determined for the antibiotic standards. The established method was applied for semi-quantitative determination of flumequine residues in milk. Matrix solid-phase dispersion was used as a pre-separation method.

Keywords: Flumequine, Thin-layer chromatography, Direct bioautography, Matrix solid-phase dispersion, Milk

INTRODUCTION

Fluoroquinolones are important synthetic antibacterial agents, which are widely used in human and veterinary treatment. They are highly active against Gram-negative and moderately active against Gram-positive

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bacteria. In many countries, fluoroquinolones are approved for use as therapeutic agents and as feed additives for food producing animals. Together with the misuse of these antibiotics in human medicine, this has led to the loss of their efficacy and to the emergence of drug-resistant bacteria. Flumequine (9-fluoro-6,7-dihydro-5-methyl-1-oxo-1H,5H-benzo[ij]quinolizine-2-carboxylic acid), belonging to fluoroquinolones, is often applied in farming of cattle, poultry, and fish (Fig. 1). It is especially effective against *Eserichia coli, Salmonella spp.* and *Pasteurella spp.* infections. If withdrawal periods are not followed, the residues of flumequine can be present in food, causing a risk to human health. According to the Polish regulations, flumequine is not allowed to be present in the food supply.[1] The European Union has set maximum residue limits (MRLs) for flumequine in bovine, ovine, and porcine muscle, liver, and kidney, at 200, 500, and 1,500 ppb, respectively, in chicken and turkey muscle, liver and kidney at 400, 800, and 1,000 ppb, respectively, and in Salmonidae muscle at 600 ppb. A very low MRL value, i.e., 50 ppb, has been established for bovine milk.[2]

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The physicochemical methods usually employed for fluoroquinolone assays are high performance liquid chromatography,[2–7] thin-layer chromatography,[8–14] and capillary electrophoresis.[15,16] High performance liquid chromatography (HPLC) is the most common method used for the analysis of flumequine and other fluoroquinolones.[2,17,18] However, when many samples should be analyzed, thin layer chromatography (TLC) seems to be more convenient. Thin layer chromatography–direct bioautography (TLC-DB) combines TLC with microbiological detection.[12,13,19–22] The developed TLC plates are dipped in a bacterial growth medium seeded with an appropriate bacterial strain. Bacteria grow directly on the TLC plate so that, not only separation, but also incubation and visualization are performed on the plate. In the previous papers, the conditions for TLC-DB of some antibiotics were established.[12,13,21,22] MSPD based on Chromosorb WAW was used as a pre-separation method for TLC-DB of enrofloxacin and ciprofloxacin residues in milk.[22]

In this paper, MSPD was used as a pre-separation method for TLC-DB of flumequine residues in milk. The TLC-DB conditions were first established for the standards.

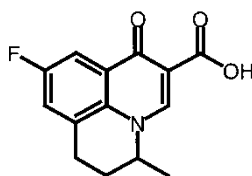

Figure 1. Structure of flumequine.

EXPERIMENTAL

Equipment and Reagents

DS sandwich chambers were purchased from Chromdes, Lublin, Poland.[23] Pre-coated silica gel TLC plates $Si60F_{254}$ were purchased from E. Merck KGaA, (Darmstadt, Germany).

Flumequine was supplied by Sigma (St. Louis, MO, U.S.A.). Hexane, dichloromethane, methanol, and acetonitrile, HPLC grade, were from Merck (Darmstadt, Germany). Ammonia (25%) and 2-propanol were purchased from P. O. Ch. (Gliwice, Poland). Chromaton N-AW 80-100 mesh was from Lachema (Brno, Czech Republic). The Chrom Biodip® Antibiotics Test Kit was purchased from E. Merck (Darmstadt, Germany).

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Preparation of Standards

The stock solution of flumequine was prepared in 0.03M NaOH at 1 mg mL^{-1}. This solution, as well as the solutions at 0.1 and 0.01 mg mL^{-1}, prepared by diluting the stock solution with 0.03M NaOH, were used for spiking the milk samples at 10, 1, 0.1 and 0.05 ppm levels, respectively. The standard solutions were prepared by diluting the stock solution with methanol or the mobile phase. All solutions were stored at $-18°C$.

Matrix Solid-Phase Dispersion

Chromaton N-AW was blended with the spiked milk sample (1 mL taken from 4 mL, spiked at a given level) and acetonitrile in mass to mass proportion 2:1:1 (2 g per 1 mL of milk and 1 mL of ACN) and put into the syringe. The sample in the cartridge was defatted with 10 mL of hexane (aspirated by the water pump) and the syringe was centrifuged for 5 min (8,400 × g). The hexane eluates were rejected. Then, 10 mL of dichloromethane was used to elute the antibiotics. The syringe was centrifuged again and both CH_2Cl_2 eluates were combined. The sample was then evaporated to dryness; the test tube was rinsed with 1 mL of dichloromethane, which was evaporated to dryness again and the residue at the bottom of the tube was dissolved in 100 μL of the mobile phase.

Sample Spotting and Development

The samples and the standards were applied to the TLC plate using a Linomat 5 Camag applicator (Muttenz, Switzerland). The mobile phase was

dichloromethane/methanol/2-propanol/25% aqueous ammonia (3:3:5:2). The plates were developed to the top and then continuously for 1 h.

Bioautography

Bioautography was performed according to the Chrom Biodip® Antibiotics Test Kit recipe.[24] One bottle of nutrient medium was mixed with 200 mL of 0.5 M Tris buffer in a 300 mL Erlenmeyer flask, adjusted to pH 7.2 with 1 M hydrochloric acid, and autoclaved for 20 min. The sterile medium was then inoculated by pipetting in the *Bacillus subtilis* spore suspension and incubated for 4 h at 37°C (incubation time was prolonged compared to that proposed by Merck).

The developed TLC plates were dried successively in air and in a vacuum desiccator. They were then immersed briefly in the microorganism (MO) solution and incubated 20 h at 37°C. After incubation, the plates were sprayed with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-solution and left at room temperature for about 30 min. Cream-white inhibition zones were observed against a purple background. The plates were dried in air and scanned for documentation. The inhibition zone areas were then measured with a planimeter.

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RESULTS AND DISCUSSION

The optimal conditions for the MSPD procedure were established earlier.[22] The spiked milk was mixed with Chromaton N-AW and acetonitrile, which causes precipitation of milk proteins. The mobile phase was found previously for the separation of six fluoroquinolones, flumequine being among them.[14] This phase, i.e., dichloromethane/methanol/2-propanol/25% aqueous ammonia (3:3:5:2) proved to be very useful for TLC-DB of flumequine. The preliminary experiments were performed for flumequine standards. The series of standards were prepared in methanol and in the mobile phase. The typical bioautograms obtained for the series of standards dissolved in the mobile phase are presented in Figs. 2 and 3. The first one presents the inhibition zones obtained for the series of standards applied in various volumes and at the same concentration. The inhibition zones on the latter bioautogram were obtained for the standards applied at a constant volume (10 μL) and at various concentrations. Corresponding spots on both plates contained the same amounts of the antibiotic. Figure 4 presents a calibration curve corresponding to Fig. 2, i.e., the function between the inhibition zone areas and logarithms of amounts contained in various volumes spotted. The areas are the means obtained from two chromatograms. The shape of the curve is exponential. The inhibition zone area depends, not only on the amount of flumequine in the spot, but also on the volume spotted—the larger the volume, the greater the

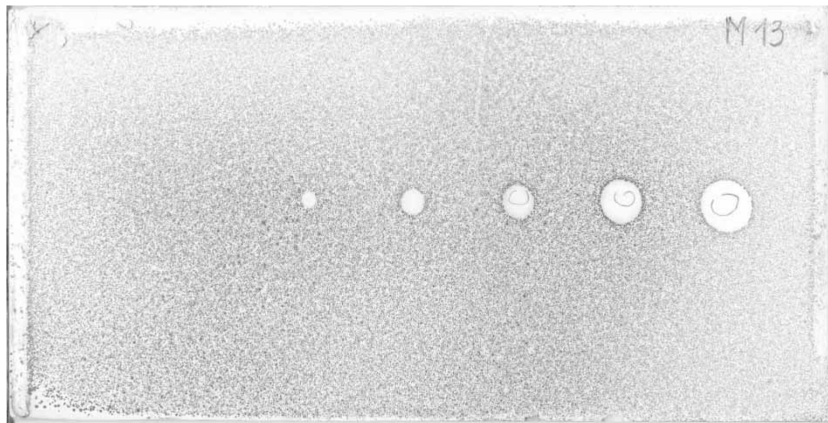

Figure 2. TLC-DB of flumequine standards. TLC $Si60F_{254}$ plate, the mobile phase: dichloromethane/methanol/2-propanol/25% aqueous ammonia (3:3:5:2). The plate was developed to the top and then continuously for about 1 h. The applied volumes, from left to right, were: 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10 μL at constant concentration (0.01 μg μL^{-1}). Band length, 1.0 mm.

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area. The exponential dependencies between the inhibition zone areas and logarithms of concentration obtained for the fixed volumes (10, 20, or 50 μL) spotted on a given plate are presented in Fig. 5. As proved earlier, the exponential shape of such plots is typical for bioautography.[13] The areas of

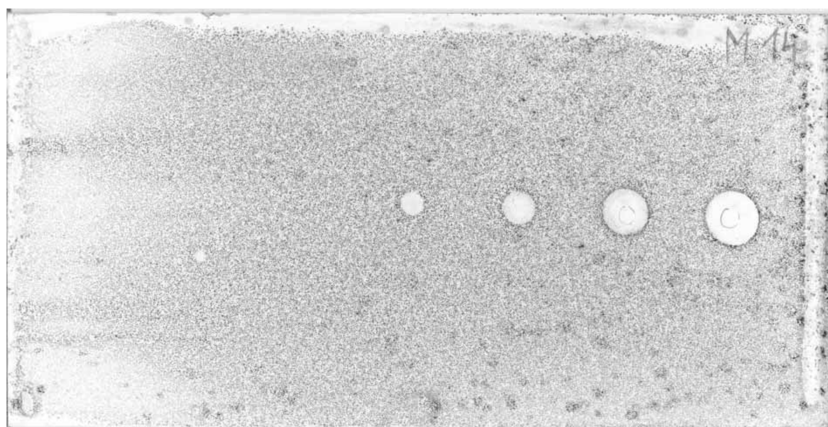

Figure 3. TLC-DB of flumequine standards. The chromatographic conditions as for Fig. 2. The applied volume was constant and equals 10 μL. From left to right, increasing concentrations of the standards: 0.1, 0.2, 0.5, 1, 2, 5, and 10 ng μL^{-1}. Band length, 1.0 mm.

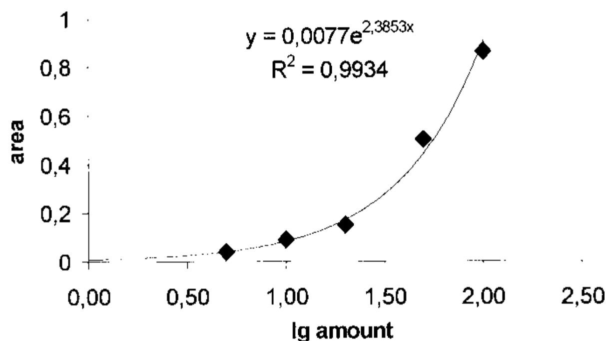

Figure 4. Areas of inhibition zones (cm^2) versus logarithm of amount contained in various volumes applied to the plate (ng).

inhibition zones were calculated on the basis of two chromatograms repeated for the same volume.

Figure 6 presents one of the chromatograms obtained for the spotted volume of 50 μL. For such a large volume applied, the limit of detection was at 0.2 ppm (0.01 μg per spot). For 20 μL, the limit of detection was at 0.5 ppm (0.01 μg per spot) and, for 10 μL at 1 ppm (again 0.01 μg per spot). Similar experiments were performed for the standards dissolved in methanol but, what was rather unexpected, the limits of detection were higher by about one order of magnitude. From that moment, all samples, both the standards and the eluates from the cartridges, were dissolved in the mobile phase. The experiments performed for the standards indicated that the MRL level of flumequine in milk, i.e., 0.5 ppm, should be reached when samples at volumes greater than 20 μL were applied.

The following experiments were performed for the spiked milk samples. The milk was spiked at various levels (10, 1, 0.1, and 0.05 ppm) with

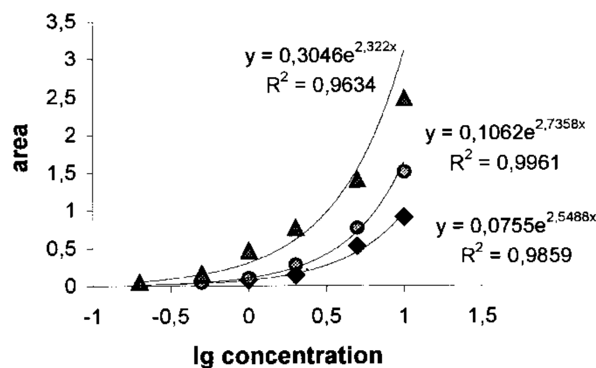

Figure 5. Areas of inhibition zones (cm^2) versus logarithm of concentration (ng μL^{-1}) for the fixed volume spotted. 10 μL – rhombus, 20 μL – circle, 50 μL – triangle.

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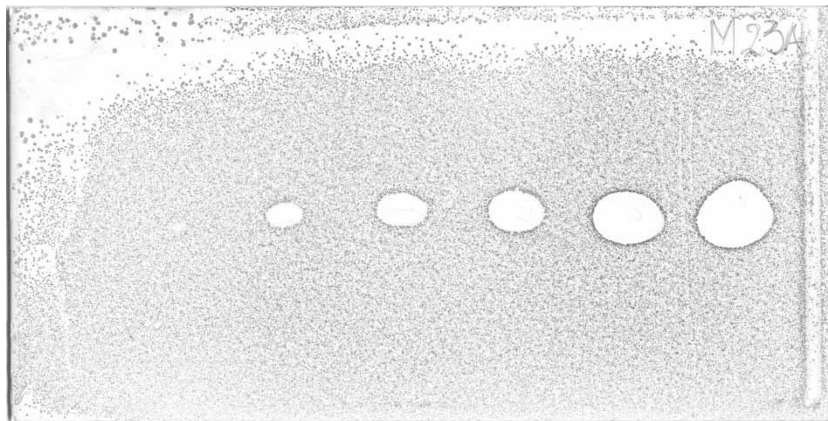

Figure 6. TLC-DB of flumequine standards. The chromatographic conditions were as for Fig. 2. The applied volume was constant and equals 50 μL. From left to right, increasing concentrations of the standards: 0.1, 0.2, 0.5, 1, 2, 5, and 10 ng μL^{-1}. Band length, 5.0 mm.

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flumequine. Three MSPD cartridges were prepared for a given concentration level and the final eluates from these three cartridges were spotted on the TLC plates, together with the standards and the blank samples, for which the same procedures, but for the net milk samples, were carried out. The plates were developed, dried, and subjected to bioautography. The inhibition zone areas were measured, compared to those of the standards, and recoveries were calculated. The mean recoveries were calculated on the basis of three inhibition zones corresponding to three eluates from three MSPD cartridges prepared from milk spiked at a given level. For the spiking levels of 10 and 1 ppm, the same eluates were spotted several times on various plates and at various volumes (from 1 to 20 μL) and then mean recoveries were calculated for each volume spotted. The eluates corresponding to the milk spiked at 0.1 and 0.05 ppm were spotted on the plate at a volume of 50 μL and one time only. All of the experiments were repeated for another portion of milk coming from another box, but from the same batch of milk.

The typical bioautogram obtained for the milk spiked with flumequine at the 1 ppm level (10 ppm in the eluates from MSPD cartridges) is presented in Fig. 7 while, for the milk spiked at the 0.05 ppm level (0.5 ppm in the eluates from MSPD cartridges) in Fig. 8.

Table 1 contains mean recoveries and means of the means obtained for the inhibition zones corresponding to a given level of concentration. As is seen, the mean recovery was about 70% for all levels of concentrations. The lower the spiking level, the larger the standard deviation. The standard deviations were not calculated for the levels 0.1 and 0.05 ppm because only two means for the two sets of three MSPD cartridges were obtained. This was

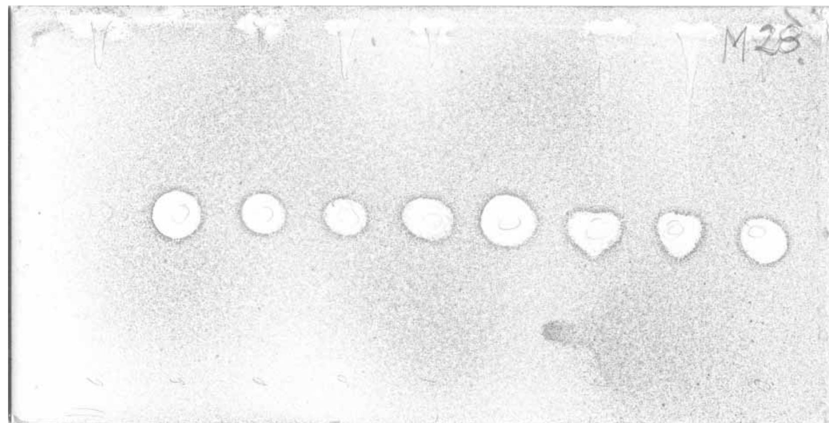

Figure 7. TLC-DB of the standards and eluates corresponding to milk spiked at the 1 ppm level. The chromatographic conditions were as for Fig. 2. Band length, 1.0 mm. From left to right: 1. 15 μL of the blank sample (the same MSPD and TLC-DB procedure as for samples 6A, 6B, and 6C but for the net milk). 2. 15 μL of 10 ppm flumequine standard. 3. 15 μL of the eluate from the cartridge denoted 6A. 4. 15 μL of the eluate from the cartridge denoted 6B (the same spiked milk portion but the second cartridge). 5. 15 μL of the eluate from the cartridge denoted 6C (the same spiked milk portion but the third cartridge). 6., 7., 8., and 9. – the same as for 2., 3., 4., and 5 respectively but for 20 μL samples applied.

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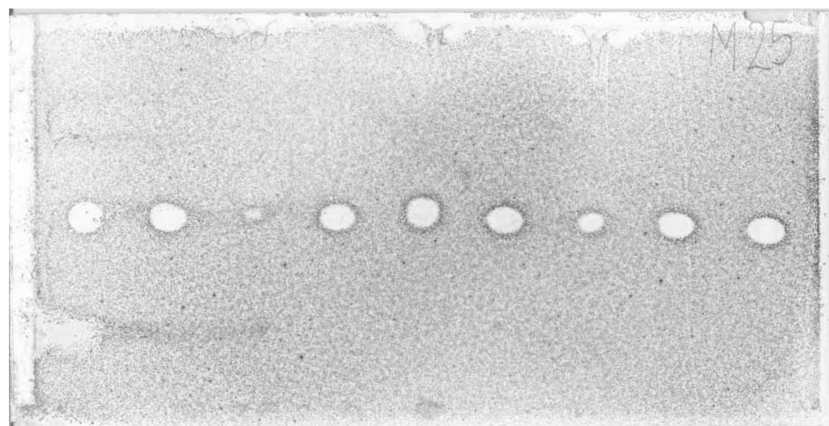

Figure 8. TLC-DB of the standards and eluates corresponding to milk spiked at 0.05 ppm level (0.5 ppm in the eluate from MSPD cartridge). The chromatographic conditions were as for Fig. 2. Band length, 5.0 mm. From left to right: 1., 2., 4., 6., 8., and 9–50 μL of 0.5 ppm standards of flumequine. 3. – 50 μL of the eluate from the cartridge denoted 4A. 5. – 50 μL of the eluate from the cartridge denoted 4B (the same spiked milk portion but the second cartridge). 7. –50 μL of the eluate from the cartridge denoted 4C (the same spiked milk portion but the third cartridge).

Table 1. Mean recoveries obtained for the inhibition zones corresponding to a given level of concentration. For each concentration level two different portions of milk were tested (another box from the same batch)

Concentration level	Mean recovery for 3 cartridges and given volume spotted	Mean of the means ± SD (RSD)
10 ppm	56.02 (1 μL)	75.15 ± 10.97 (14.6)
	82.91 (2 μL)	
	66.88 (3 μL)	
	77.19 (4 μL)	
	64.75 (5 μL)	
	74.07 (1 μL)	
	79.44 (2 μL)	
	91.38 (3 μL)	
	83.74 (5 μL)	
1 ppm	43.68 (5 μL)	74.82 ± 19.73 (26.36)
	83.77 (10 μL)	
	81.75 (15 μL)	
	49.29 (5 μL)	
	84.34 (10 μL)	
	89.0 (15 μL)	
	91.94 (20 μL)	
0.1 ppm	113.0 (50 μL)	76.0
	39.77 (50 μL)	
0.05 ppm	70.0 (50 μL)	79.0
	88.0 (50 μL)	

related to the very low concentration of flumequine in the milk samples and, what follows, to the necessity of spotting large volumes of the MSPD eluates on the plate, i.e., 50 μL. It was impossible to repeat spotting because the total volume of the sample was 100 μL. As a consequence, the recoveries were calculated on the basis of only three inhibition zones relating to three MSPD cartridges. The inhibition zones corresponding to such large spots are not reproducible and means are calculated with a large error. Nevertheless, it is evident that the above method enables screening of milk samples for flumequine content at the MRL level.

CONCLUSIONS

1. The method presented enables semi-quantitative estimation of flumequine residues in milk at MRL level.
2. The detection level for flumequine depends on the spotted volume.
3. It is necessary to spot very large volumes for samples containing flumequine at the MRL level.

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4. A lower detection level and better reproducibility could probably be achieved if bioautography were done with different species of bacteria, e.g., Gram-negative *Escherichia coli* instead of Gram-positive *Bacillus subtilis*, used in the Biodip test.

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