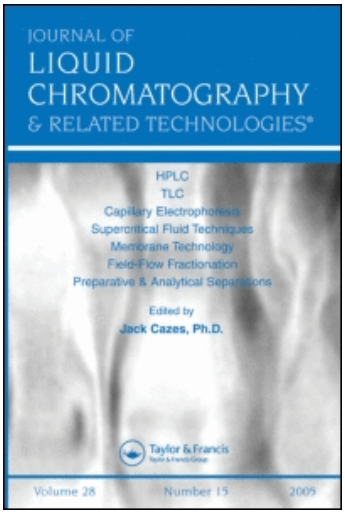

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I. M. Choma[a]; A. Choma[b]; K. Staszczuk[a]
[a] Department of Chemical Physics and Physicochemical Separation Methods, M. Curie-Sklodowska University, Lublin, Poland [b] Department of General Microbiology, M. Curie-Sklodowska University, Lublin, Poland

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DETERMINATION OF FLUMEQUINE IN MILK BY THIN-LAYER CHROMATOGRAPHY-BIOAUTOGRAPHY

I. M. Choma,[1,*] A. Choma,[2] and K. Staszczuk[1]

[1]Department of Chemical Physics and Physicochemical Separation Methods, M. Curie-Sklodowska University, M. Sklodowska Sq. 3, 20-031 Lublin, Poland
[2]Department of General Microbiology, M. Curie-Sklodowska University, Akademicka St. 19, 20-033 Lublin, Poland

ABSTRACT

Fluoroquinolones are synthetic antibiotics widely used in human and animal medical treatment. Flumequine, belonging to this group, is often used in medical treatment and prevention in livestock. It is also added to feed or water to obtain better weight gain. Many physicochemical techniques have been reported for the assay of fluoroquinolones. Thin-layer chromatography (TLC) is a method of choice for analysis because it is cheap and simple, provides high sample throughput, and requires limited sample pre-treatment. Thin-layer chromatography-bioautography (TLC-B) is the screening technique, which combines TLC with microbiological detection resulting in enhanced sensitivity. In this paper, a simple TLC-B method for determination of flumequine in milk is described. The sample pre-treatment is

*Corresponding author. E-mail: ichoma@hermes.umcs.lublin.pl

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performed directly on the plate. Microbiological detection directly on the plate allows for enhanced sensitivity of the method.

Key Words: Flumequine; Milk; TLC-B; TLC-Bioautography

INTRODUCTION

Fluoroquinolone antibiotics are a relatively new group of synthetic antibiotics derived from 3-quinolone carboxylic acid. They are widely used in the treatment of both human and veterinary diseases. Flumequine (9-fluoro-6,7-dihydro-5-methyl-1-oxo-1H,5H-benzo[ij]quinolizine-2-carboxylic acid) is frequently used in fish, poultry, and cattle husbandry for the treatment and prevention of diseases. It is also used, as a feed or water additive, to enhance weight gain of livestock. According to the Polish regulations, flumequine is not allowed to be present in the food supply.[1] Yet, flumequine is still available for veterinary use and can be detected in meat or milk when the withdrawal period for the drug is not abided by. High concentrations of the antibiotic can be found in milk when it is added to prolong the freshness of the milk.

The methods for determining antibiotics in food are based on microbiological,[2–3] immunochemical,[3–5] and physicochemical principles.[3,6] The most popular methods belong to the latter group and are chromatographic techniques, mainly liquid chromatography including high-performance liquid chromatography (HPLC)[3,6–9] and thin-layer chromatography (TLC).[10–12]

Thin-layer chromatography-bioautography (TLC-B) is the screening method, which combines TLC with microbiological detection. The developed TLC plates are placed on or dipped in a bacterial growth medium seeded with an appropriate bacterial strain. The location of zones of growth inhibition allows for the information about antibiotic residues.[13–16]

In this paper simple TLC method described elsewhere[17,18] was combined with bioautography to detect flumequine in milk.

EXPERIMENTAL

Equipment and Reagents

DS sandwich chambers[19,20] were purchased from Chromdes, Lublin, Poland. Pre-coated silica gel TLC plates $Si60F_{254}$ 10×20 cm, $Si60F_{254}$ 10×20 cm with concentrating zone, HPTLC $Si60F_{254}$ and HPTLC $Si60F_{254}$ were purchased from E. Merck KGaA, Darmstadt, Germany. Hexane, acetone, chloroform, methanol, citric acid, hydrochloric acid, and TRIS, reagent grade,

were purchased from P. O. Ch. Gliwice, Poland. Flumequine was supplied by Polfa, Tarchomin, Poland. Chrom Biodip® Antibiotics Test Kit was obtained from E. Merck, Darmstadt, Germany.

Methods

Preparation of Antibiotics Solutions

A 0.1 g portion of flumequine was weighed accurately into a 10-mL volumetric flask, dissolved in 9 mL of 1% Na_2CO_3 and then diluted to the volume with water. The working solution was the mixture, prepared by the dilution of 100 μL of each stock-solution in 9.9 mL of methanol to produce 0.1 mg/mL standards. These standards were diluted 10-fold again to produce 0.01 mg/mL standards. Milk (2% fat) was fortified with the stock solutions of the antibiotic.

Sample Spotting and Development

The standard solutions or milk spiked with flumequine were applied to the TLC plate using a Hamilton microsyringe (Bonaduz, Switzerland). The milk samples were injected on the TLC plates into the middle of special regions of a trapezoidal shape created by the incision into the plate's concentrating zone.[17,18] The plates with concentrating zones were pre-developed successively with hexane and acetone to remove lipid fractions from the milk samples. They were then developed with one of the mobile phases, i.e., 0.05 M citric acid–methanol–2-propanol (1 : 3 : 1) or chloroform–acetone (9 : 1). The standards injected on to the plates without concentrating zones (without spots of milk samples) were only developed with one of the mobile phases.

Bioautography

Bioautography was performed according to Chrom Biodip® Antibiotics Test Kit recipe. 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 2 h at 35°C.

The developed TLC plates were immersed briefly in the microorganism (MO) solution and incubated overnight at 28°C. Then the plates were sprayed with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-solution and incubated for about 30 min. Yellow inhibition zones were seen against a purple background.

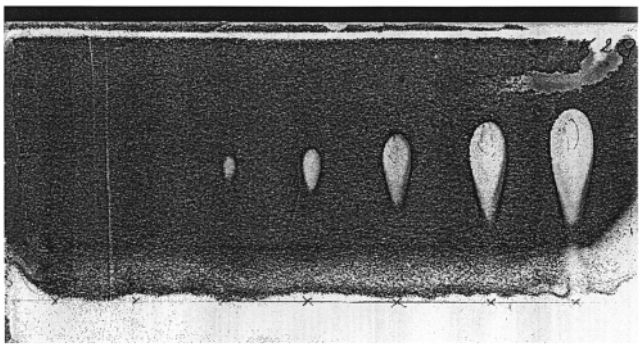

Figure 1. TLC-B of flumequine standards on $Si60F_{254}$ plate. The amounts injected from left to right: 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 μL at concentration of 0.01 μg/μL; e.g., 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1 μg of flumequine per spot. Developed with the phase: 0.05 M citric acid–methanol–2-propanol (1 : 3 : 1).

RESULTS AND DISCUSSION

In previous papers, a simple screening TLC method for determining tetracycline antibiotics and flumequine in milk was established.[17,18] In the present paper, the TLC method similar to the above mentioned, was used to isolate flumequine from milk. The spots of milk were injected into the middle of the specially prepared regions of the trapezoidal shape created by incisions into the

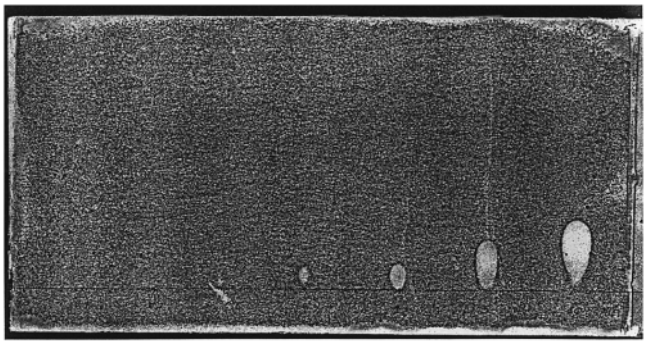

Figure 2. TLC-B of flumequine standards on $Si60F_{254}$ plate. The amounts injected from left to right: 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 μL at concentration of 0.01 μg/μL; e.g., 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1 μg of flumequine per spot. Developed continuously for 1.5 h with the phase chloroform–acetone (9 : 1).

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plate's concentrating zone. The plate was pre-developed with hexane, which removes non-polar milk lipids and then with acetone which dissolves the more polar lipid fractions. The plate was then developed with the proper solvent system. The defatted milk proteins remained on the surface of the concentrating zone while flumequine, separated in this way from the milk matrix, migrated freely. The developed chromatographic plates were immersed and inoculated with *B. subtilis* medium and incubated to permit the growth of bacteria. After spraying with MTT, dehydrogenases of living microorganisms convert tetrazolium salt into purple formazan. Flumequine spots appeared as clear areas on a purple background.

Several mobile phases were tested, but only two of them were useful both for isolation of flumequine from milk and for microbial detection. The phases of choice were 0.05 M citric acid–methanol–2-propanol (1 : 3 : 1) and chloroform–acetone (9 : 1).

Figures 1 and 2 present TLC-B of flumequine standards injected from 0.001 μg to 0.1 μg, on to TLC plates and developed with the mobile phases

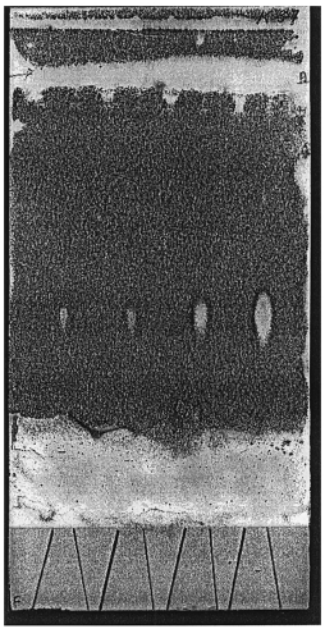

Figure 3. TLC-B of 1 μL milk samples spiked with flumequine, injected into the middle of the special regions of the trapezoidal shape created by incisions into the concentrating zone of $Si60F_{254}$ plate. The amounts of flumequine per spot from left to right: 0.001, 0.01, 0.02, 0.05 μg. The plate was successively developed with: hexane, acetone and the mobile phase: 0.05 M citric acid–methanol–2-propanol (1 : 3 : 1).

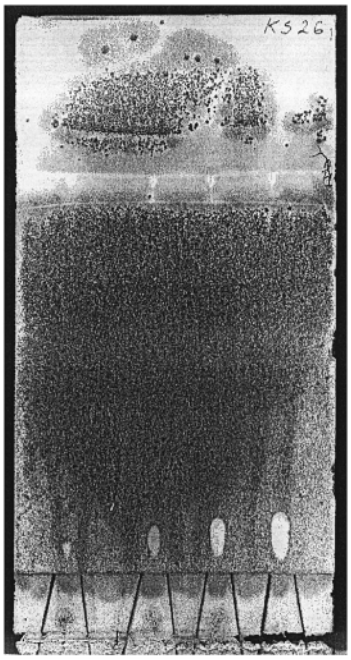

Figure 4. TLC-B of 5 μL milk samples spiked with flumequine, injected into the middle of the special regions of the trapezoidal shape created by incisions into the concentrating zone of $Si60F_{254}$ plate. The amounts of flumequine per spot from left to right: 0.005, 0.025, 0.05, 0.1 μg. The plate was continuously developed with: hexane, acetone and the mobile phase chloroform–acetone (9 : 1) (continuously for 1.5 h).

0.05 M citric acid–methanol–2-propanol (1 : 3 : 1) and chloroform–acetone (9 : 1), respectively. The limit of detection for both phases equals 0.005 μg and is 10 times lower than for UV detection.[17] Figures 3 and 4 are the TLC chromatograms of flumequine isolated from milk samples spiked with different amounts of this antibiotic. Both plates were pre-developed successively with hexane and acetone and developed with 0.05 M citric acid–methanol–2-propanol (1 : 3 : 1) (Fig. 3) and chloroform–acetone (9 : 1) (Fig. 4). As can be seen, the limit of detection (0.001 μg) is lower than that obtained for the standards, probably because of the concentrating properties of the injection zone. It is possible to increase this level by an order of magnitude by injecting 10 μL of milk instead of 1 μL (greater than 10 μL volumes exceed the capacity of the concentrating zone). Then the limit of detection of 0.1 ppm can be reached.

Figure 5 shows an HPTLC chromatogram of milk samples spiked with different amounts of flumequine, predeveloped with hexane, followed by acetone,

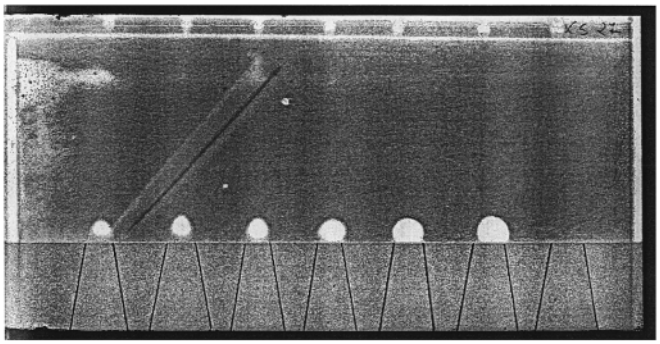

Figure 5. TLC-B of 5 μL milk samples spiked with flumequine and injected into the middle of special regions of the trapezoidal shape created by incision into the concentrating zone of HPTLC $Si60F_{254}$ plate. The amounts of flumequine per spot from left to right: 0.005, 0.025, 0.05, 0.1, 0.25, 0.50 μg and additionally 5 μL sample of pure milk. The plate was successively developed with: hexane, acetone and the phase chloroform–acetone (9 : 1) (continuously for 1.5 h).

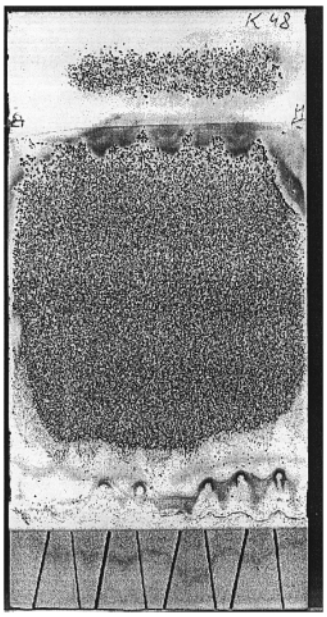

Figure 6. Blank TLC-B chromatogram of 1 μL milk samples injected into the concentrating zone of $Si60F_{254}$ plate developed successively with: hexane, acetone and the phase: 0.05 M citric acid–methanol–2-propanol (1 : 3 : 1).

and developed with the mobile phase chloroform–acetone (9 : 1). Additionally, the spot of pure milk was injected. Any compound of antibacterial activity was eluted from the milk in this system. This was confirmed by a blank chromatogram of milk samples developed in the same way. A smooth layer of bacteria covered all the plate (the figure is not shown). The blank chromatogram of milk samples developed with the mobile phase 0.05 M citric acid–methanol–2-propanol (1 : 3 : 1) shows the zone of bacterial growth inhibition at the bottom of the plate (Fig. 6). The same phenomenon was observed in Fig. 3. This is probably connected with the demixing of the mobile phase, which is stripped of the more polar component, e.g., citric acid, at the front. Too high a concentration of citric acid at the bottom of the plate disturbed the growth of the bacteria. Fortunately, this zone of inhibition does not influence the detection of flumequine spots.

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