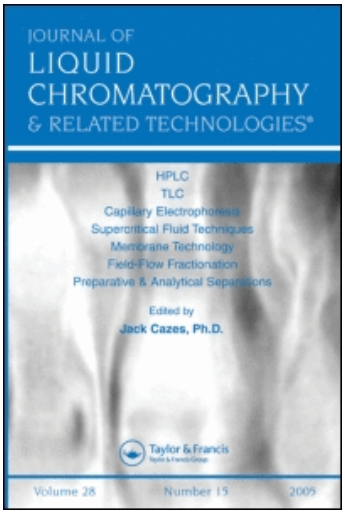

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Matrix Solid-Phase Dispersion Combined with Thin-Layer Chromatography-Direct Bioautography for Determination of Enrofloxacin and Ciprofloxacin Residues in Milk

Irena Choma[a]; Iwona Komaniecka[b]

[a] Department of Chromatographic Methods, M. Curie-Skłodowska University, Lublin, Poland [b] Department of General Microbiology, M. Curie-Skłodowska University, Lublin, Poland

To cite this Article Choma, Irena and Komaniecka, Iwona(2005) 'Matrix Solid-Phase Dispersion Combined with Thin-Layer Chromatography-Direct Bioautography for Determination of Enrofloxacin and Ciprofloxacin Residues in Milk', Journal of Liquid Chromatography & Related Technologies, 28: 16, 2467 — 2478

To link to this Article: DOI: 10.1080/10826070500189406

URL: http://dx.doi.org/10.1080/10826070500189406

Journal of Liquid Chromatography & Related Technologies®, 28: 2467–2478, 2005

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500189406

Matrix Solid-Phase Dispersion Combined with Thin-Layer Chromatography–Direct Bioautography for Determination of Enrofloxacin and Ciprofloxacin Residues in Milk

Irena Choma
Department of Chromatographic Methods, M. Curie-Skłodowska University, Lublin, Poland

Iwona Komaniecka
Department of General Microbiology, M. Curie-Skłodowska University, Lublin, Poland

Abstract: Enrofloxacin is a common veterinary antibiotic, which can be found as a residue in milk, together with its main metabolite, ciprofloxacin. Chromosorb WAW was used as a sorbent for matrix solid-phase dispersion of milk samples spiked with these antibiotics. This pre-separation method was combined with thin-layer chromatography-direct bioautography to obtain semi-quantitative results. Various modes of the procedure were tested and the one giving the best recovery of the antibiotics from milk was chosen.

Keywords: Enrofloxacin, Ciprofloxacin, Thin-layer chromatography–direct bioautography, Matrix solid-phase dispersion

INTRODUCTION

In our previous paper, a novel version of matrix solid-phase dispersion (MSPD) for isolation of doxycycline and flumequine in milk was established.[1] It was

Address correspondence to Irena Maria Choma, Department of Chromatographic Methods, M. Curie-Skłodowska University, M. Skłodowska Sq. 3, 20-031 Lublin, Poland. E-mail: ichoma@hermes.umcs.lublin.pl

proven that bare siliceous sorbents with a low surface area could be used for MSPD of milk samples before HPLC analysis instead of expensive C_{18} sorbents. The MSPD procedure, introduced in 1989 by S.A. Baker as a process for the disruption and extraction of viscous, semi-solid, or solid samples, involves blending of the sample with a solid support material, usually silica with bonded C_{18} chains.[2,3] In our paper, three siliceous sorbents were applied, i.e., kieselguhr, wide-pore silica gel, and Chromosorb WAW; the last one proved to be optimal.

Enrofloxacin and ciprofloxacin belong to fluoroquinolones, chemotherapeutics widely used in medical treatment because of their good antibacterial activity and pharmacokinetic properties.[4] Enrofloxacin is used in veterinary medicine, while its main metabolite, ciprofloxacin, is a popular human antibiotic.[4,5] Both these drugs can be present as residues in milk.[6,7] Hence, there is a need for assays capable of determining and distinguishing between them. High performance liquid chromatography (HPLC) is, undoubtedly, the most common method used in the analysis of enrofloxacin and ciprofloxacin, as well as other fluoroquinolones.[6–10] However, when many samples have to be analyzed, thin-layer chromatography (TLC) seems to be more convenient. Our intention was to establish the MSPD method giving the best recovery of enrofloxacin and ciprofloxacin from milk. Thus, various versions of this procedure were tested, i.e., various milk to Chromosorb ratios, various solvents for elution, and various additives. TLC was used to compare the recoveries obtained for various MSPD methods of milk samples spiked with enro- and ciprofloxacin.

Thin-layer chromatography-bioautography (TLC-B) 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 and size of growth inhibition zones allow for the information about the kind and quantity of antibiotics. In so-called direct bioautography (TLC-DB), bacteria grow directly on the TLC plate, so that not only separation but also incubation and visualization are performed on the plate.[11,12] In the recent paper, the conditions for semi-quantitative TLC-DB of enrofloxacin and ciprofloxacin were established.[13]

In the present paper, MSPD based on Chromosorb WAW was used as a pre-separation method for TLC-DB of enrofloxacin and ciprofloxacin residues in milk.

EXPERIMENTAL

Equipment and Reagents

DS sandwich chambers were purchased from Chromdes, Lublin, Poland.[14] Pre-coated silica gel TLC plates $Si60F_{254}$ were purchased from E. Merck KGaA, (Darmstadt, Germany).

Enrofloxacin and ciprofloxacin were supplied by Sigma (St. Louis, MO, U.S.A.). Hexane, dichloromethane, methanol, and acetonitrile HPLC grade were from Merck (Darmstadt, Germany). Ammonia (25%), acetic acid (80%), and 2-propanol were purchased from P.O.Ch. (Gliwice, Poland). Chromosorb WAW 80–100 mesh was from Johns-Manville (Denver, Colorado U.S.A.). The Chrom Biodip® Antibiotics Test Kit was purchased from E. Merck, (Darmstadt, Germany).

Preparation of Standards

The stock solution of the mixture of enrofloxacine and ciprofloxacine was prepared in 0.03 M NaOH at 1 mg mL^{-1} of each. It was stored at $-18^{\circ}C$. The standard solution was prepared by diluting the stock solution with methanol to obtain a concentration of 0.1 mg mL^{-1}. The milk samples were spiked with the stock solutions.

Matrix Solid-Phase Dispersion

Chromosorb WAW was blended with the spiked milk sample in mass to mass proportion 2 : 1 (2 g per 1 mL) 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 (8400 × 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.

Sample Spotting and Development

The samples and the standards were applied to the TLC plate using a Hamilton microsyringe (Bonaduz, Switzerland). The mobile phase was dichloromethane/methanol/2-propanol/25% aqueous ammonia 3 : 3 : 5 : 2.

Bioautography

Bioautography was performed according to the 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 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 a vacuum desiccator. They were then immersed briefly in the microorganism (MO) solution and incubated overnight at 28°C. After incubation, the plates were sprayed with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-solution and incubated 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.

RESULTS AND DISCUSSION

In our previous papers, we found the best mobile phase for the separation of enrofloxacin and ciprofloxacin as well as TLC-DB conditions.[13,15] In this paper, preliminary measurements were carried out to find the best conditions for the MSPD procedure. Both enrofloxacin and ciprofloxacin belong to the so-called 7-piperazinylquinolones. They can be in anionic, zwitterionic, or cationic form, depending on pH of aqueous solutions, since they possess both carboxylic and amine groups (see Figure 1). In neutral pH they are zwitterionic. The influence of neutral, acidic, or basic conditions, and of various deproteinization agents on the MSPD procedure, were tested with regard to the best recovery of enrofloxacin and ciprofloxacin from milk. The following procedures were performed (see "Experimental" for details):

1. 2 g of Chromosorb WAW + 1 mL of milk eluted with:
   a. 10 mL of hexane
   b. 10 mL of dichloromethane/methanol/25% aqueous ammonia (5 : 4 : 1). The sorbent to milk ratio equaled 2 : 1 was found earlier.[1]
2. 2 g of Chromosorb WAW + 1 mL of milk + 1 mL of 25% aqueous ammonia eluted with:
   a. 10 mL of hexane
   b. 10 mL of dichloromethane
3. 2 g of Chromosorb WAW + 1 mL of milk + 1 mL of 80% acetic acid eluted with:
   a. 10 mL of hexane
   b. 10 mL of dichloromethane
4. 2 g of Chromosorb WAW + 1 mL of milk + 1 mL of methanol + 1 mL of 80% acetic acid eluted with:
   a. 10 mL of hexane
   b. 10 mL of dichloromethane
5. 2 g of Chromosorb WAW + 1 mL of milk + 1 mL of acetonitrile eluted with:
   a. 10 mL of hexane
   b. 10 mL of dichloromethane

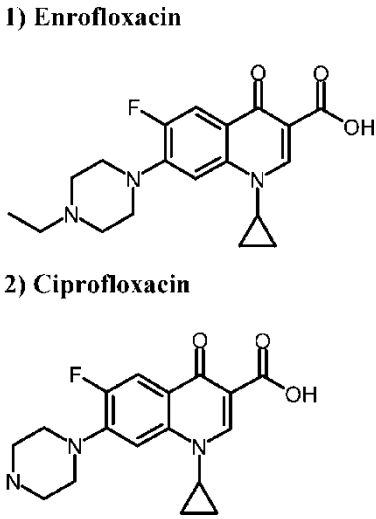

Figure 1. Structures of 1) enrofloxacin and 2) ciprofloxacin.

The milk was spiked at 10 ppm level with both antibiotics. Each procedure was repeated twice and the eluates from two 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 then recoveries were calculated. Table 1 contains mean recoveries obtained for a few inhibition zones corresponding to a given procedure.

As can be seen from Table 1, the basic conditions led to low recoveries when ammonia was used as an addition to the eluting solvent, as well as when it was added directly to the spiked milk (procedure 1 and 2). The addition of acetic acid to the milk samples results in high recovery of enroflox-

Table 1. Mean recoveries, standard deviations SD and relative standard deviations RSD obtained for the inhibition zones corresponding to a given procedure (each procedure repeated twice and the eluates spotted 2 or 3 times)

Number of procedure (Number of repetitions)	Enrofloxacin mean recovery ± SD (RSD (%))	Ciprofloxacin mean recovery ± (SD, RSD (%))
1. (6)	23.3 ± 8.4 (36.2)	15.0 ± 3.8 (25.5)
2. (4)	24.3 ± 12.3 (50.5)	18.0 ± 10.0 (55.6)
3. (4)	116.0 ± 71.0 (61.2)	29.3 ± 25.0 (84.6)
4. (4)	76.0 ± 4.0 (5.3)	95.0 ± 30.0 (31.6)
5. (4)	88.3 ± 16.8 (19.1)	56.3 ± 12.5 (22.2)

acin and low recovery of ciprofloxacin (procedure 3). The standard deviation is very high, especially for ciprofloxacin, which can be associated with the error in the inhibition zone area estimation. This is basically due to the presence of an unknown antibacterial substance in the blank sample, which has R_F value similar to that of ciprofloxacin. Hence, its inhibition zone area should be subtracted from that of ciprofloxacin, causing additional error. There is also another inhibiting substance, which gives an additional spot in the bioautogram above the spot of enrofloxacin (cf. Figure 2).

The high recoveries of both enrofloxacin and ciprofloxacin were obtained for procedure 4. However, an additional zone of inhibition appears at the start, besides the spot at the R_F value similar to that of ciprofloxacin. The substance co-eluting with ciprofloxacin appears in all the procedures, despite procedure 5. For this reason, this procedure was finally chosen for further experiments. In the preliminary experiments described above, the eluates from the cartridges were evaporated to dryness and then reconstituted in 100 μL of dichloromethane. This could cause an additional error because of the changes in the antibiotic concentrations due to the high volatility of this solvent. Hence, dichloromethane was replaced with methanol as was described in the "Experimental" section. Procedure 5 was repeated and, additionally, the following procedures were tested:

6. 2 g of Chromosorb WAW + 1 mL of milk + 1 mL of acetonitrile + 1 mL of 80% acetic acid eluted with:
   a. 10 mL of hexane
   b. 10 mL of dichloromethane
7. 2 g of Chromosorb WAW + 1 mL of milk + 1 mL of acetone + 1 mL of 80% acetic acid eluted with:
   a. 10 mL of hexane
   b. 10 mL of dichloromethane
8. 2 g of Chromosorb WAW + 1 mL of milk eluted with:
   a. 10 mL of hexane
   b. 10 mL of dichloromethane

The milk was spiked again at 10 ppm level. Each MSPD procedure was repeated three times.

Table 2 contains mean recoveries, standard deviations, and relative standard deviations calculated for each of the MSPD procedures. The eluates (from three cartridges for a given procedure) were spotted on the same plate and at the same volume. MSPD procedures 6 and 7, both involving an organic solvent (acetonitrile or acetone) and acetic acid, give a high standard deviation and, in case of procedure 7—low recovery. Additionally, in the case of procedure 7, an unknown inhibiting substance of R_F value greater than that of enrofloxacin appears at the corresponding bioautograms (as in Figure 2 corresponding to procedure 3). Procedure 8 is repeatable especially for enrofloxacin, which is illustrated by the standard deviation for

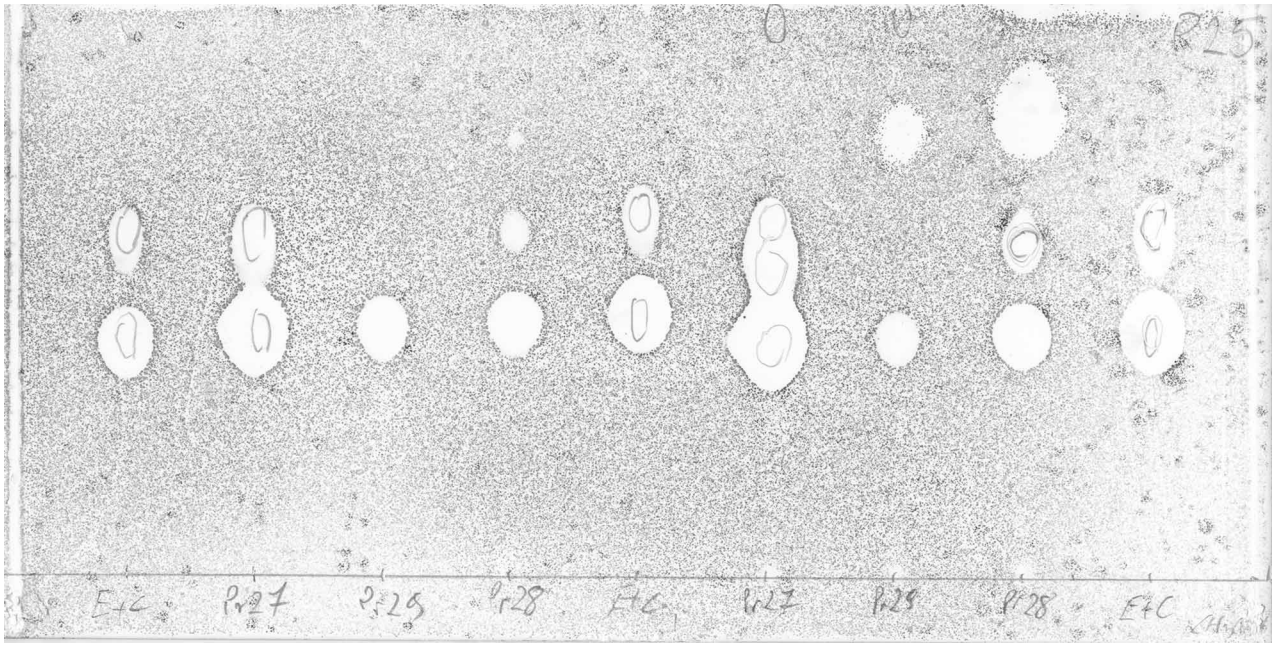

Figure 2. TLC-DB of the standards and eluates obtained according to MSPD procedure 3. Enrofloxacin—upper spots, ciprofloxacin—lower spots. TLC $Si60F_{254}$ plate, the mobile phase dichloromethane/methanol/2-propanol/25% aqueous ammonia 3 : 3 : 5 : 2. The plate was developed to the end and then continuously for about 2 h. The plate number is "p25". From left to right: 1 μL of the standard of enro- and ciprofloxacin (each at 0.1 mg mL^{-1}); 1 μL of the eluate from the cartridge; 1 μL of the blank sample; 1 μL of the eluate from the cartridge (the same procedure but another cartridge); 1 μL of the standard of enro- and ciprofloxacin (each at 0.1 mg mL^{-1}); 6, 7, 8, 9—the same as for 2, 3, 4, 5, respectively, but 2 μL samples were applied.

Table 2. Mean recoveries, standard deviations SD and relative standard deviations RSD obtained for the given procedure (each procedure repeated three times, the applied volume 1 μL)

Number of procedure (Number of repetitions)	Enrofloxacin mean recovery ± SD (RSD (%))	Ciprofloxacin mean recovery ± SD (RSD (%))
5′. (3)	88.6 ± 4.9 (5.6)	80.7 ± 2.0 (2.4)
6. (3)	76.2 ± 24.3 (31.9)	59.5 ± 10.5 (17.7)
7. (3)	57.1 ± 23.3 (40.8)	48.3 ± 16.2 (33.6)
8. (3)	86.2 ± 0 (0)	40.3 ± 7.4 (18.4)

this drug (zero value means that inhibitions zone areas corresponding to three eluates from three MSPD cartridges are the same). However, a very low recovery of ciprofloxacin was obtained.

The recoveries calculated for procedure 5 are also presented in Table 2 (5′ indicates that methanol was used instead of dichloromethane). The mean recovery for both antibiotics was excellent and a low standard deviation was obtained. In order to estimate the repeatability of the method, the same procedure was repeated for three next cartridges (5″) (Figure 3). The eluates from MSPD cartridges 5′ and 5″ were applied at volumes ranging from 1 to 4 μL and on various plates. For each set of recoveries (for a given procedure, a given plate and at a given volume of eluate) mean values were calculated. They are reported in Table 3. The total mean recovery, calculated from all results obtained for procedure 5′ and 5″ equals 84.2% for enrofloxacin (SD = 8.5%, RSD = 10.1%) and 71.7% for ciprofloxacin (SD = 11.7%, RSD = 16.3%).

Figure 4 presents dependencies between logarithm of the volume applied and the area of inhibition for enrofloxacin and ciprofloxacin. These typical bioautography plots exhibit good correlation. Figure 5 presents comparison of the inhibition zone areas of enrofloxacin and ciprofloxacin for three eluates obtained with procedure 5″ and applied on two different TLC plates. A comparison of the eluates obtained with procedures 5′ and 5″, respectively, is presented in Figure 6. As is seen, the inhibition zone areas are very reproducible for enrofloxacin while those for ciprofloxacin are not. This appears to be associated with both non reproducible recovery of ciprofloxacin from different cartridges and probably with not adequate reproducibility of the TLC-direct bioautography (compare 5″A/15 and 5″A/16 in Figure 5).

The method works well for a rather high level of enrofloxacin and ciprofloxacin in milk, i.e., 10 ppm. This high concentration was convenient to make the choice among various MSPD conditions, but is too high compared with the MRL level (100 ppb for the sum of enrofloxacin and ciprofloxacin residues in milk). Such a level could be achieved with larger spotting volumes and/or increasing the milk to sorbent ratio. In the near future, we will address these problems. However, the present results clearly demonstrate that the MSPD

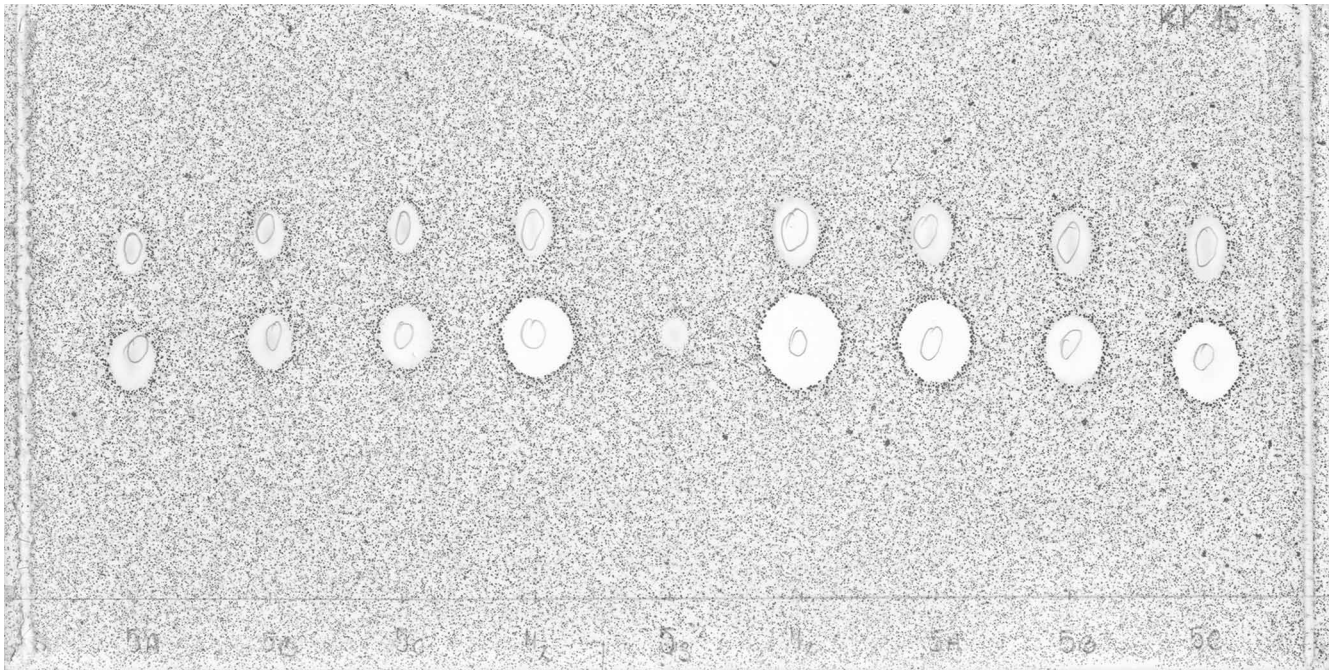

Figure 3. TLC-DB of the standards and eluates obtained according to MSPD procedure 5″. Enrofloxacin—upper spots, ciprofloxacin—lower spots. The chromatographic conditions same as for Figure 2. The plate number is "15". From left to right: 1 μL of the eluate from the cartridge denoted 5″A; 1 μL of the eluate from the cartridge denoted 5″B (the same procedure but the second cartridge); 1 μL of the eluate from the cartridge denoted 5″C (the same procedure but the third cartridge); 1 μL of the standard of enro- and ciprofloxacin (each at 0.1 mg mL^{-1}); 2 μL of the blank sample denoted 5″S; 2 μL of the standard of enro- and ciprofloxacin (each at 0.1 mg mL^{-1}); 7, 8, 9—the same as for 1, 2, 3, respectively, but now 2 μL samples were applied.

Table 3. Mean recoveries obtained for procedure 5′ and 5″(each procedure repeated three times), for given spotted volume and given bioautogram (the bioautograms indicated by the TLC plates order numbers)

Procedure number/spotted volume [μL]/the plate number	Enrofloxacin Mean recovery ± SD (RSD) [%]	Ciprofloxacin Mean recovery ± SD (RSD) [%]
5′/1/9	88.6 ± 4.9 (5.6)	80.7 ± 2.0 (2.4)
5′/2/9	74.5 ± 7.9 (10.6)	83.7 ± 3.2 (3.9)
5″/1/15	88.6 ± 4.9 (5.6)	62.1 ± 12.3 (19.7)
5″/2/15	74.5 ± 3.3 (4.4)	70.4 ± 14.6 (20.7)
5″/1/16	85.7 ± 0 (0)	64.4 ± 10.4 (16.2)
5″/2/16	88.5 ± 18 (20.4)	81.9 ± 5.4 (6.7)
5″/3/17	85.3 ± 3.5 (4.1)	68.8 ± 9.4 (13.7)
5″/4/17	87.9 ± 4.4 (5.0)	61.6 ± 13.5 (21.9)
The mean of the means	84.2 ± 5.7 (6.8)	71.7 ± 8.6 (11.9)

method can be easily combined with the HPLC technique to give quantitative results.

CONCLUSIONS

Chromosorb WAW can be used as a sorbent for matrix solid-phase dispersion of enrofloxacin and ciprofloxacin residues in milk. This pre-separation method can be combined with thin-layer chromatography-direct bioautography to obtain semi-quantitative results. The best recoveries of the antibiotics from milk were obtained when milk was mixed not only with Chromosorb WAW

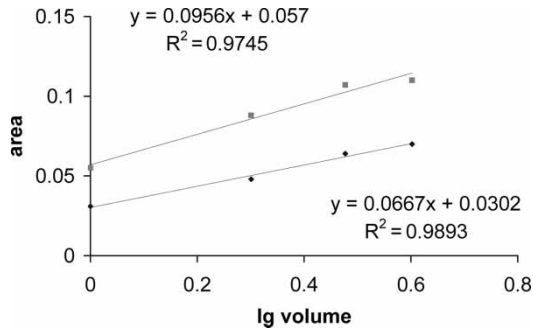

Figure 4. Areas of inhibition zones [cm^2] (the means obtained for the procedure 5″, plates 15 and 17) versus logarithm of eluate volume applied onto the plate [μL]. Rhombus—enrofloxacin, square—ciprofloxacin.

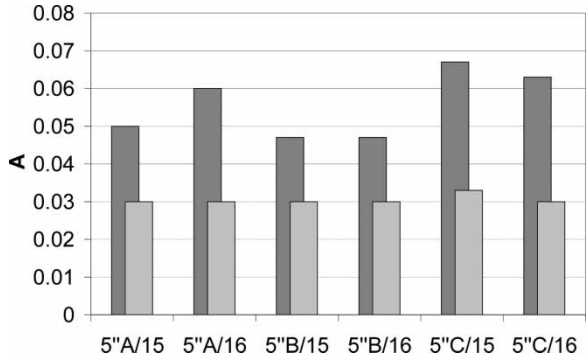

Figure 5. The inhibition zone areas of enrofloxacin and ciprofloxacin [cm^2] for three eluates obtained with procedure 5″ (denoted 5″A, 5″B, and 5″C) applied on two different TLC plates (denoted "15" and "16"). 5″A/15 means that eluate from the cartridge 5″A was applied on the plate "15", while 5″B/16 means the same eluate was applied on the plate "16". Light gray stands for enrofloxacin, dark gray for ciprofloxacin.

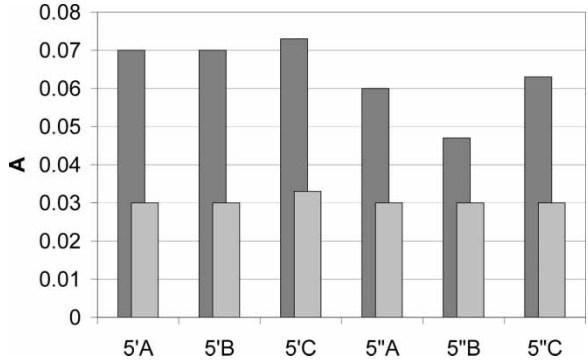

Figure 6. The inhibition zone areas of enrofloxacin and ciprofloxacin [cm^2] for three eluates obtained with procedure 5′ and 5″. A, B, C stands for the cartridges from the same series, obtained for the same procedure. Light gray stands for enrofloxacin, dark gray for ciprofloxacin.

but also with acetonitrile, which caused precipitation of milk proteins. The developed pre-separation method can be combined with other analytical methods, for instance HPLC. The great advantage of TLC-DB compared to HPLC is a possibility of testing many samples at the same time.

REFERENCES

1. Choma, I.; Pilorz, K. A novel application of matrix solid-phase dispersion for determination of doxycycline and flumequine residues in milk. J. Liq. Chrom. & Rel. Technol. **2004**, *27*, 2143–2151.

2. Barker, S.A. Applications of matrix solid-phase dispersion in food analysis. J. Chromatogr. A **2000**, *880*, 63–68.
3. Barker, S.A. Matrix solid-phase dispersion (MSPD). In *Solid-Phase Extraction: Principles, Techniques, and Applications*; Simpson, N.J.K., ed.; Marcel Dekker, Inc.: New York, 2000; 361–380.
4. Lambert, H.P.; O'Grady, F.W. *Antibiotics and Chemotherapy*, 6th Ed.; Longman: London, 1992.
5. Allen, D.G.; Pringle, J.K. *Handbook of Veterinary Drugs*, 2nd Ed.; Lippincott-Raven: Philadelphia, 1998.
6. Tyczkowska, K.L.; Voyksner, R.D.; Kevin, L.A.; Papich, M.G. Simultaneous determination of enrofloxacin and its primary metabolite ciprofloxacin in bovine milk and plasma by ion-pairing liquid chromatography. J. Chromatogr. B **1994**, *658*, 341–348.
7. Cinquina, A.L.; Roberti, P.; Giannetti, L.; Longo, F.; Draisci, R.; Fagiolo, A.; Brizioli, N.R. Determination of enrofloxacin and its metabolite ciprofloxacin in goat milk by high-performance liquid chromatography with diode-array detection. Optimization and validation. J. Chromatogr. A **2003**, *987*, 221–226.
8. Hernández-Arteseros, J.A.; Barbosa, J.; Compañó, R.; Prat, M.D. Analysis of quinolone residues in edible animal products. Review. J. Chromatogr. A **2002**, *945*, 1–24.
9. Carlucci, G. Analysis of fluoroquinolones in biological fluids by high-performance liquid chromatography. Review. J. Chromatogr. A **1998**, *812*, 343–367.
10. Belal, F.; Al-Majed, A.A.; Al-Obaid, A.M. Methods of analysis of 4-quinolone antibacterials. Review. Talanta **1999**, *50*, 765–786.
11. Botz, L.; Nagy, S.; Kocsis, B. Detection of microbiologically active compounds. In *Planar Chromatography*, 1st Ed.; Nyiredy, Sz., ed.; Springer: Budapest, 2001; 489–516.
12. Eymann, R.; Hauck, H.E. Chrom Biodip® Antibiotics—a high performance bioautographic test kit. In *Proceedings—Planar Chromatography 2000*; Lillafüred: Hungary, 2000; 67–75.
13. Choma, I.; Choma, A.; Komaniecka, I.; Pilorz, K.; Staszczuk, K.J. Semi-quantitative estimation of enrofloxacin and ciprofloxacin by thin-layer chromatography-direct bioautography. J. Liq. Chromatogr. & Rel. Technol. **2004**, *27*, 2071–2085.
14. Dzido, T.H. New modification of the horizontal sandwich chamber for TLC: variants of development technique. J. Planar Chromatogr. **1990**, *3*, 199–200.
15. Choma, I. TLC separation of fluoroquinolones: searching for better selectivity. J. Liq. Chromatogr. & Rel. Technol. **2003**, *26*, 2673–2685.

Received December 15, 2004
Accepted January 11, 2005
Manuscript 6634A