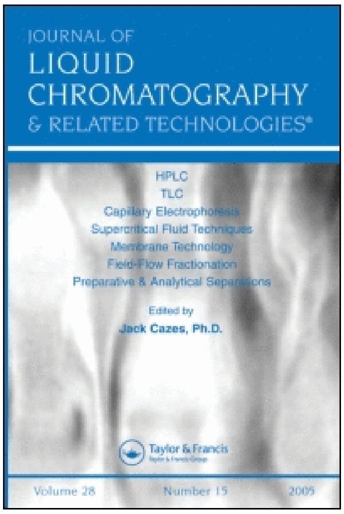

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A Novel Application of Matrix Solid-Phase Dispersion for Determination of Doxycycline and Flumequine Residues in Milk

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ABSTRACT

A novel version of matrix solid-phase dispersion (MSPD) for isolation of doxycycline (DC) and flumequine (FL) in milk was established. The MSPD procedure involves blending of sample with solid support material, usually silica with bonded C18 chains. In the work presented, bare siliceous sorbents were applied i.e., kieselguhr, wide-pore silica gel and Chromosorb WAW. The sorbents were blended with the spiked milk using sorbent to milk ratio 4 : 1 w/w. The "pastes," formed in that way, were put into the syringe and defatted with hexane. Then, dichloromethane was used to elute FL. The mixture of 0.5 M citric acid/methanol/2-propanol (1 : 3 : 1) or 0.5 M citric acid/methanol (1 : 9) were used

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to elute DC. RP-HPLC and NP-HPLC were used for simultaneous determination of the antibiotics. It was possible to detect the antibiotics in milk at 100 ppb level. Chromosorb proved to be the optimal MSPD sorbent.

Key Words: Matrix solid-phase dispersion; Doxycycline; Flumequine; Residues; Milk.

INTRODUCTION

Doxycycline (DC), tetracycline antibiotic, and flumequine (FL) from fluoroquinolones, are frequently used in veterinary treatment and animal husbandry. They are also used to enhance the weight gain of livestock as feed or water additives. When withdrawal periods are not obeyed, unsafe antibiotic residues, or their metabolites, may be present in edible products e.g., in milk, eggs, and meat.[1,2] The traces of antibiotics in food can be dangerous because of direct toxicity, and because of the emergence of antibiotic-resistant bacteria.[3] Therefore, it is important to develop reliable methods to detect such residues in food.[4] According to the Polish regulations, neither FL nor DC is allowed to be present in the food supply.[5] Maximum residue limits (MRLs) in milk, established by EU, equals 50 and 100 ppb for FL and DC, respectively.[1,2,6,7] Isolation of tetracyclines and fluoroquinolones, and their separation from matrices is complicated, since both groups have a propensity to form chelate complexes with metal ions and sample matrix proteins, and because they interact strongly with silanol groups of siliceous sorbents. The thin-layer chromatography (TLC) method of isolation from milk, and separation of DC and FL, was described previously.[8,9] The method consists of defatting milk spotted into the TLC plate with hexane and/or acetone, followed by the antibiotics' separation with a proper mobile phase. The milk proteins remained at the concentration zone of the plate and did not disturb the chromatographic process.

The main idea of our present paper was to check if the similar method, based on retaining milk proteins on siliceous sorbents, could be used for sample preparation prior to HPLC analysis of FL and DC. Matrix solid-phase dispersion (MSPD), introduced in 1989 by Baker as a process for the disruption and extraction of viscous, semi-solid, or solid samples, seemed to be a method of choice.[7,10] Our method was based on MSPD in a novel version, that is using siliceous sorbents with low surface areas (similar to the sorbent of the concentrating zone of the TLC plate) instead of commonly used for MSPD C18 sorbents.

In the present paper, MSPD based on siliceous sorbents was used as a preseparation method for high performance liquid chromatography determination of DC and FL residues in milk. RP-HPLC and NP-HPLC methods were established for their simultaneous analysis. The recoveries obtained for various sorbents and at various concentration levels were compared.

EXPERIMENTAL

Reagents and Materials

DC hydrochloride and FL were obtained from Sigma (St. Louis, MO). Methanol, dichloromethane, and acetonitrile HPLC grade were from Merck (Darmstadt, Germany). 2-Propanol, citric, and oxalic acids analytical grade were purchased from P.O.Ch. (Gliwice, Poland). The following siliceous sorbents were used: kieselguhr Merck (Darmstadt, Germany), silica gel 1500 Å Amicon (USA), and Chromosorb WAW Macherey-Nagel (Düren, Germany). Water used for HPLC was redistilled and purified in a Milli-Q system Millipore (Milford, MA).

Preparation of Standards

The stock solutions of FL were prepared in 0.03 M NaOH, while solutions of DC were prepared in water, both at 10 mg/mL. They were stored at −18°C. The standard solutions for calibration plots were prepared by diluting the stock solutions with 0.5 M aqueous citric acid solution/methanol 1 : 1 to obtain concentrations of 0.1, 0.01, 0.001, and 0.0001 mg/mL. The milk samples were spiked directly with the stock solutions or with the stock solutions diluted with water.

MSPD

The sorbents (kieselguhr, silica gel, or Chromosorb WAW) were blended with the spiked milk sample in mass to mass proportion 4 : 1 or 2 : 1 and put into the syringe. The sample in the cartridge was defatted with 10 mL of hexane. Then, 10 mL of dichloromethane was used to elute FL. The eluate was evaporated to dryness and dissolved in 0.5 mL of 0.5 M citric acid aqueous solution/methanol (1 : 1). The mixture of 0.5 M citric acid/methanol/2-propanol (1 : 3 : 1) or 0.5 M citric acid/methanol (1 : 9) was used to elute DC (10 mL again). The eluate volume was reduced by evaporation to 1 mL and then injected into RP-HPLC system. In the case of NP-HPLC, which serves for preliminary measurements at high concentration levels, the eluates from the cartridge were not evaporated and injected directly into NP-HPLC system.

Chromatography

The HPLC system consisted of Beckman 110 B HPLC pump (San Ramon, USA), Rheodyne 7125 injector with 20 μL loop (Cotati, USA), variable-wavelength 155 UV/VIS Gilson detector (Middleton, USA) and CSW32 DataApex acquisition system (Prague, Czech Republic).

Preliminary measurements concerning the usefulness for MSPD of three siliceous sorbents (i.e., kieselguhr, silica gel, and Chromosorb WAW) were done in the NP-HPLC system, using 150 mm × 4.6 mm Ultrasphere, 5 μm, Beckman column (San Ramon, USA) and 0.5 M citric acid/methanol/2-propanol (1 : 3 : 1) as the mobile phase, while RP-HPLC was done using 250 mm × 4.6 mm, Zorbax, 5 μm, SB-C18 column (Agilent Technologies, Wilmington, USA) and acetonitrile/methanol/oxalic acid aqueous solution pH 1.3 (30 : 20 : 50) as the mobile phase. FL was detected at 315 nm, DC at 350 nm. The detector was programmed to switch the wavelength at 5 min of run. The flow rate was set at 1 mL/min.

Physicochemical Measurements

Specific surface areas and mean pore diameters were measured by means of the ASAP 2405N Micromeritics (Norcross, GA).

RESULTS AND DISCUSSION

HPLC separation conditions for simultaneous analysis of DC and FL were established earlier.[11] The sample preparation method was based on MSPD using siliceous sorbents with low surface area instead of the ones commonly used for MSPD i.e. C18 sorbents. The sorbents (kieselguhr, wide-pore silica gel, or Chromosorb WAW) were blended with the spiked milk, in mass to mass proportion 4 : 1 or 2 : 1, and put into the syringe. The physicochemical characteristics of the sorbents applied, i.e., the specific surface areas and mean pore diameters, are collected in Table 1.

The preliminary experiments were done by NP-HPLC using the mobile phase 0.5 M citric acid/methanol/2-propanol (1 : 3 : 1), similar to that used in TLC experiments. This phase was also used for elution of DC from the cartridge. The mean recoveries are presented in Table 2. The worst recoveries

Table 1. Specific surface areas and mean pore diameters of the sorbents.

Sorbent	Specific surface area (m^2/g)	Mean pore diameter (Å)
Chromosorb WAW	1	50
Kieselguhr	5	50
Silica gel 1500	33	1000

were obtained for kieselguhr, which was not surprising, as this sorbent contains many metal impurities strongly retaining the antibiotics. Good recoveries were obtained with Chromosorb WAW and wide-pore silica gel. Chromosorb was chosen for further experiments because of economical reasons.

We found that good reproducibility of the peak areas of antibiotic standards can be obtained when they are dissolved in 0.5 M citric acid aqueous solution/methanol (1 : 1) instead of water or methanol. Calibration for DC and FL in a RP-HPLC system was done for a wide range of concentrations (see Experimental), so linear regression was performed in the log–log scale. Good correlation was obtained for the logarithm of peak area vs. logarithm of amount injected. For nine injections of DC and FL standards at four concentration levels, we found standard errors of 0.021 and 0.039, while squared correlation coefficient were equal to 0.9997 and 0.9989, respectively. The calibration curves are presented in Fig. 1.

The recoveries were measured at three concentration levels: 100, 50, and 0.1 ppm (cf. Table 3). The 50 ppm level was obtained by spiking twice as big a volume of milk as for the 100 ppm level with the same amount of the antibiotics (that is with 50 μg). It seemed, that the proportion of the sorbent to the sample 4 : 1, typical for MSPD, could be changed to 2:1 without any loss of recovery. It was also verified, that the phase used previously for

Table 2. The medians of recovery of FL and DC isolated from milk by means of MSPD using different siliceous sorbents.

	Cartridge	
	DC	FL
Chromosorb WAW—2 g	109 (4)	128 (3)
Kieselguhr—2 g	58 (2)	66 (2)
Silica gel 1500—2 g	110 (5)	93 (3)

Note: DC and FL—200 ppm; Milk—0.5 mL. Number of experiments in brackets. The eluates were analyzed by NP-HPLC.

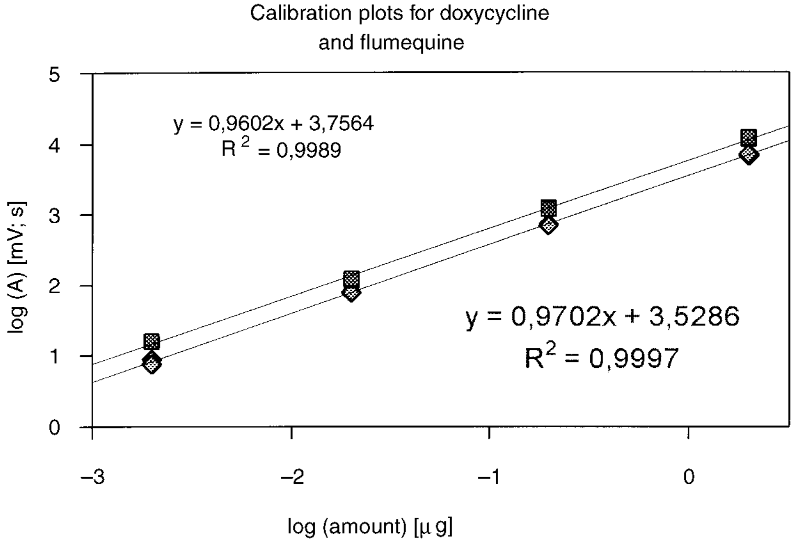

Figure 1. Calibration curves of doxycycline and flumequine. Square for doxycycline, rhombus for flumequine.

eluting DC from the cartridge i.e. 0.5 M citric acid/methanol/2-propanol (1 : 3 : 1) can be replaced with 0.5 M citric acid/methanol (1 : 9), which was easier to evaporate. Figure 2 presents chromatograms of 0.5 M citric acid/ MeOH (1 : 9) extract (containing DC) and CH_2Cl_2 extract (containing FL) of milk spiked at the 100 ppm level. The recoveries were good for higher

Table 3. The medians of recovery of FL and DC isolated from milk by means of MSPD.

	Cartridge	
	DC	FL
DC and FL—100 ppm, Milk–0.5 mL	77 (4)	82 (4)
DC and FL—50 ppm, Milk–1 mL	93 (2)	73 (2)
DC and FL–0.1 ppm, Milk–0.5 mL	140 (4)	213 (4)

Note: Chromosorb—2 g. Number of experiments in brackets. The eluates were analyzed by RP-HPLC.

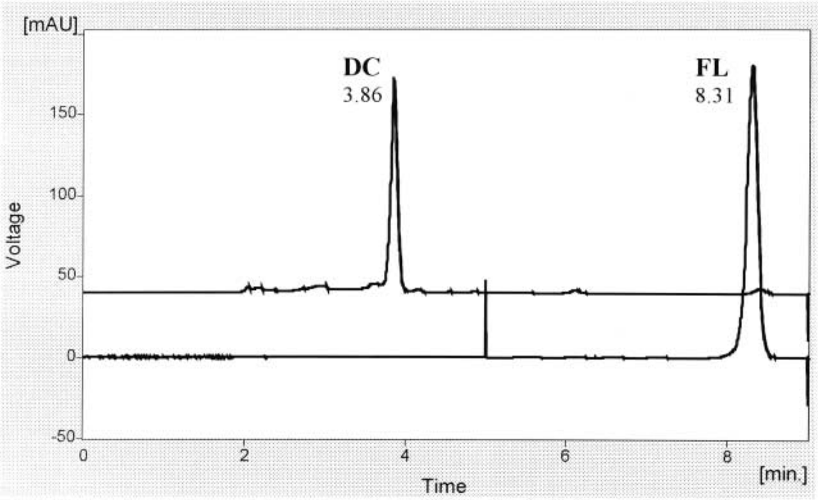

Figure 2. Chromatograms of: CH_2Cl_2 extract (containing FL) evaporated to the dryness and reconstituted in 0.5 mL 0.5 M citric acid/MeOH (1 : 1); 0.5 M citric acid/MeOH (1 : 9) extract (containing DC) evaporated to 1 mL (the shifted up chromatogram). Both extracts were obtained for the same milk sample spiked at 100 ppm level with FL and DC and mixed with Chromosorb WAW in 4 : 1 proportion.

spiking levels, but they were too high, especially for FL, at 0.1 ppm. Still, it is possible to detect both DC and FL at this level and to estimate their amount. Using 1 mL instead of 0.5 mL of milk, it should be possible to obtain the MRL level not only for DC but also for FL i.e., 50 ppb. The problem with calculating precise recoveries of FL at the 0.1 ppm level, is probably due to the appearance of the peak at the retention time of FL on the blank chromatograms (cf. Fig. 3). It may be a peak of some milk constituent, or what is more probable, it may be a residue of FL in milk. It is necessary to explain what kind of substance this could be. We are going to address ourselves to further measurements at this low level, testing more samples, different kinds of milk, and different proportions of sorbent to milk.

CONCLUSIONS

It was proven that siliceous sorbent with low surface area can be used for MSPD of milk samples instead of expensive C18 sorbents. The described procedure presents a simple and cheap method of milk sample preparation for isocratic HPLC analysis. At the moment, the procedure can be treated as

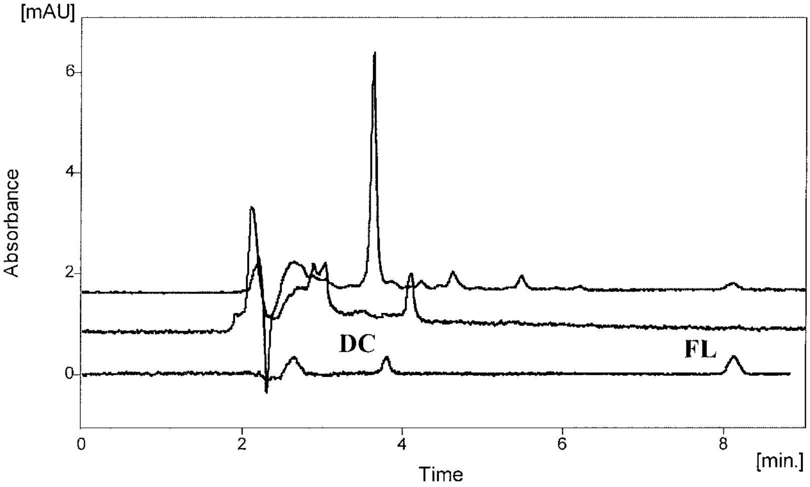

Figure 3. From the bottom to the top chromatograms of: (1) DC and FL standards at 0.1 ppm level (350 nm switched at 5 min to 315 nm); (2) 0.5 M citric acid/MeOH (1 : 9) extract from the cartridge filled with Chromosorb WAW and milk (4 : 1), evaporated to 1 mL—blank (350 nm); (3) CH_2Cl_2 extract from the cartridge filled with Chromosorb WAW and milk (4 : 1), evaporated to the dryness and reconstituted in 0.5 mL 0.5 M citric acid/MeOH (1 : 1)—blank (315 nm).

a screening method, which enables detection of DC and FL in milk at the MRL level and estimation of their amounts. In the future, we plan to address ourselves to the problem of improving sensitivity and accuracy to put the method on fully quantitative grounds.

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