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IDENTIFICATION OF ANTIBIOTIC RESIDUES IN MILK BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A scheme has been devised that makes it possible to separate and identify, by means of thin-layer chromatography, the 14 different antibiotic residues in milk which are, besides penicillin, the most widely used in mastitis control: cloxacillin, dihydrostreptomycin, tetracycline, oxytetracycline, chlortetracycline, chloramphenicol, neomycin, novobiocin, bacitracin, erythromycin, oleandomycin, ampicillin, streptomycin and oxacillin. The limits of detectability of the antibiotics studied vary between 0.1 and 3 μg/ml, with the exception of neomycin the minimum detectable concentration of which is 15 μg/ml.

INTRODUCTION

In addition to penicillin, other antibiotics are increasingly used for the intramuscular or intramammary treatment of mastitis in cows. Microbiological methods (coagulation, bromocresol purple and *Bacillus calidolactis* tests) enable inhibitory substances in milk to be relatively easily traced[1], but do not permit distinction to be made between antibiotics and other inhibitory substances. Only the presence of penicillin can positively be determined by using the enzyme penicillinase[2].

The identification of antibiotics can be very useful, as it may establish whether or not the milk contains antibiotic residues, which is especially important when juridical problems arise in this connection. A large number of techniques are available for the separation of the various antibiotics, the most widely used being paper chromatography[3–5], high-tension electrophoresis[6,7], gas chromatography[8,9], mass spectrometry[8,9] and thin-layer chromatography[10–15]. The results obtained by many of these methods are, however, of little use for the identification of antibiotic residues in milk, because in many instances use is made of aqueous solutions of antibiotics in high concentration or because the separation of only a restricted number of antibiotics is required.

The aim of this study was to develop an identification method for antibiotic residues in milk, which occur in relatively low concentrations. The basis of this work was the study made by Frères and Valdebouze[11], who proposed a method for the

separation and identification of about ten antibiotics in feed. A few of their extraction methods were adapted for the tests on milk, and certain solvents, test organisms and culture media were also adopted for use.

Because of its rapid and simple application, thin-layer chromatography, in conjunction with bioautographic visualization, was chosen in order to achieve the identification of 14 different antibiotics. According to the IDF working group "Mastitis", these antibiotics are, besides penicillin, the most widely used in mastitis control: cloxacillin, dihydrostreptomycin, tetracycline, oxytetracycline, chlortetracycline, chloramphenicol, neomycin, novobiocin, bacitracin, erythromycin, oleandomycin, ampicillin, streptomycin and oxacillin.

Penicillin was not taken into consideration, because it can be specifically traced in the *B. calidolactis* test by using the enzyme penicillinase[2]. By adding penicillinase to the milk sample, this antibiotic is decomposed, and therefore does not interfere with the identification of the other antibiotics.

MATERIALS AND METHODS

Reagents

The following analytical grade reagents were used: acetone, acetonitrile, ammonia (at least 25%), ammonium chloride, hydrochloric acid (1 *N*), chloroform (purified on an alumina column), citric acid, dipotassium hydrogen phosphate, disodium hydrogen phosphate, ethanol, glycerol, methanol, monopotassium dihydrogen phosphate, sodium hydrogen carbonate and pentane.

Phosphate/hydrogen carbonate buffer (pH 8) was prepared by dissolving dipotassium hydrogen phosphate (16.73 g) plus monopotassium dihydrogen phosphate (0.523 g) and sodium hydrogen carbonate (20 g) in water to give 1000 ml and citrate/phosphate buffer (pH 3.7), by dissolving citric acid (1.39 g) plus disodium hydrogen phosphate (1.21 g) in water to give 1000 ml. As impregnation liquid, the mixture citrate/phosphate buffer (pH 3.7)–glycerol (19:1) was used. The solvents were (A) chloroform–acetone–impregnation liquid (5:5:2); (B) ethanol–water–ammonia (8:1:1); (C) methanol–chloroform (9:1); (D) methanol–acetone (3:2); and (E) methanol–ammonium chloride (3%) (7:3).

Thin-layer plates coated with three kinds of adsorbents were used: Kieselguhr F_{254} (Merck 5738), silica gel 60 (Merck 5721) and cellulose (Merck 5716). Before use, the Kieselguhr plate was impregnated with the above impregnation liquid. The silica gel plate was activated for 1 h at 110°.

The test organisms were *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633, *Micrococcus flavus* ATCC 10240 and *Sarcina lutea* ATCC 9341. The antibiotic preparations were supplied by the Laboratory of Standards and Pharmacopoeia (Belgian Ministry of Health, Family Welfare and Environment). Table I gives the list of preparations used, their respective potencies, and their minimum concentrations detectable (see Results and discussion).

Extraction methods

Extraction I. Acetone (26 ml) is added to 13 ml of a milksample and heated in a water bath to approx. 55°; 26 ml of pentane and 26 ml of pre-heated acetonitrile (approx. 50°) are then added. The mixture is stirred for 1 min and filtered through a

TABLE I

POTENCY AND MINIMUM DETECTABLE CONCENTRATIONS OF THE ANTIBIOTIC PREPARATIONS USED

Antibiotic	*Potency (I.U./mg)*	*Minimum detectable concentration (µg/ml)*
Cloxacillin	910*	1
Dihydrostreptomycin	736	1
Tetracycline	954	0.3
Oxytetracycline	877	0.3
Chlortetracycline	922	0.3
Chloramphenicol	1,000	1
Neomycin	658	15
Novobiocin	835	1
Bacitracin	64	2
Erythromycin	825	0.2
Oleandomycin	830	0.3
Ampicillin	740	0.1
Streptomycin	707	3
Oxacillin	885*	0.5

* µg/mg.

glass filter (P_1), the beaker and glass filter being rinsed with the mixture methanol–1 *N* HCl (9:1).

After separation and draining off, the acetone layer is evaporated under vacuum down to *ca.* 1 ml, which is then diluted with the mixture methanol–1 *N* HCl (9:1) to a final volume of 2 ml. The supernatant, after centrifugation (1 h, 2600 *g*), is extract I.

Extraction II. As for extraction I, except that the beaker and glass filter are rinsed twice with methanol–water (1:1), and that the acetone layer after evaporation is diluted to the final volume of 2 ml with the mixture methanol–phosphate/hydrogen carbonate buffer (pH 8) (1:1). Part of the supernatant after centrifugation (1 h, 2600 *g*) is extract II.

Extraction III. Two drops of ethanol and 2 ml of chloroform are added to the remaining volume of supernatant obtained during extraction II. After shaking, the chloroform layer is drained off. The upper phase is re-extracted three times with 2 ml of chloroform and the combined chloroform extracts are evaporated under vacuum down to *ca.* 2 ml, which represents extract III.

Extraction IV. Acetone (26 ml) is added to 13 ml of a milk sample in an erlenmeyer flask and heated to approx. 55°; 26 ml of pentane and 26 ml of preheated acetonitrile (approx. 50°) are then added. This mixture is filtered through a glass filter (P_1) and the filtrate discarded.

The residue in the erlenmeyer flask is rinsed with 5 ml of water and 2 ml of the mixture water–1 *N* HCl (9:1) are added. This mixture is transferred on to the glass filter. The erlenmeyer flask and the residue on the glass filter are washed four times with 2 ml of water. The filtrate obtained is then filtered until a clear liquid is obtained. The filtrate is evaporated under vacuum down to *ca.* 1 ml. The supernatant after centrifugation (1 h, 2600 *g*) is extract IV.

Chromatography

Chromatography is performed according to the scheme given in Table II. Besides the extract, aqueous solutions of the pure antibiotics are applicated as a reference.

TABLE II

CHROMATOGRAPHIC SCHEME

Thin-layer plate	*Adsorbent*	*Extraction*	*Amount of sample applicated (µl)*	*Solvent*	*Test organism*
1	Kieselguhr	I	30	A	*B. cereus*
2	Silica gel	I	30	B	*M. flavus*
3	Silica gel	II	30	C	*S. lutea*
4	Silica gel	II	30	C	*B. subtilis*
5	Silica gel	III	30	D	*M. flavus*
6	Cellulose	IV	10	E	*B. cereus*

Bioautography

The detection systems most frequently used in thin-layer chromatography are UV absorption, UV fluorescence, application of colouring reagents or mineral acids and bioautography. The last of these technique seems to be the most appropriate for the identification of antibiotic residues: it is usually more sensitive than the chemical methods, and, moreover, only the biologically active components are detected.

A plastic frame (inner dimensions 202 × 202 × 5 mm) is placed on a glass plate (250 × 250 × 3 mm); 100 ml of culture medium (45°), previously inoculated with a 20-h old culture of the test organism (approx. 3%), are then poured into the frame. Table III shows the composition and the pH of the culture media.

After development, the thin-layer plate is air dried and placed on the solidified medium. After diffusion of the antibiotics in the culture medium (about 15 min), the plate is carefully removed. When using *B. cereus* as the test organism, a second layer of non-inoculated culture medium is applied. Another glass plate is placed on the plastic frame and the whole is incubated for 20 h at 35°.

TABLE III

COMPOSITION OF THE DIFFERENT CULTURE MEDIA

Culture medium	*Test organism*			
	B. cereus	*B. subtilis*	*M. flavus*	*S. lutea*
Peptone	6.0 g	—	6.0 g	5.0 g
Tryptone	—	5.0 g	4.0 g	—
Beef extract	1.5 g	—	1.5 g	1.5 g
Yeast extract	3.0 g	2.5 g	3.0 g	1.5 g
NaCl	—	—	—	3.5 g
Glucose	—	1.0 g	1.0 g	—
Agar	10.0 g	15.0 g	10.0 g	10.0 g
Water	1000 ml	1000 ml	1000 ml	1000 ml
pH	8.7	7.0	8.5	8.3

RESULTS AND DISCUSSION

Table IV shows the R_F values obtained with the antibiotics on the different plates. These values are, however, only indicative; as mentioned under *Chromatography*, besides the extract, aqueous solutions of the pure antibiotics are applicated as a reference. The identification is possible by comparing the R_F values obtained with the extract and those obtained with the aqueous solutions.

TABLE IV

R_F VALUES OF THE ANTIBIOTICS ON THE DIFFERENT PLATES

Antibiotic	*Plate*					
	*1**	*2*	*3*	*4*	*5*	*6*
Cloxacillin	—	—	0.70	0.70	—	—
Dihydrostreptomycin	—	—	—	—	—	0.40
Tetracycline	0.35	0	0	0	0	—
Oxytetracycline	0.20	0	0	0	0	—
Chlortetracycline	0.60	0	0	0	0	—
Chloramphenicol	0	0.70	0.65	0.65	0.80	—
Neomycin	—	—	—	—	—	0
Novobiocin	—	0.80	0.80	0.80	0.80	—
Bacitracin	—	0.45	—	—	—	—
Erythromycin	0.20	0.70	0.35	0.35	0.25	—
Oleandomycin	0.20	0.70	0.35	—	0.25	—
Ampicillin	0.35	—	0.50	—	—	—
Streptomycin	—	—	—	—	—	0.50
Oxacillin	0	—	0.60	0.60	—	—

* The R_F values in this column were obtained by running the plate twice.

This table clearly shows that the 14 antibiotics investigated can be identified by the proposed chromatography scheme (*cf.* Table II). The minimum concentrations of the antibiotics still detectable after extraction and chromatography, are given in Table I.

These data indicate that the technique used is less sensitive only for neomycin (minimum detectable concentration 15 μg/ml). The limits of detectability of the other antibiotics vary between 0.1 and 3 ppm; these values closely correspond to the sensitivity of the *B. calidolactis* test[1].

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