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Analysis of chloramphenicol residues in swine tissues and milk: comparative study using different screening and quantitative methods

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ABSTRACT

Both screening and quantitative methods for chloramphenicol residues in swine tissues and milk were compared, using samples from animals treated with chloramphenicol. For screening purposes a previously developed streptavidin-biotin enzyme-linked immunosorbent assay and a commercially available immunochemical card test were used. For quantitative purposes two previously developed high-performance liquid chromatographic procedures were applied using antibody-mediated clean-up and solid-phase extraction. Some improvements in both methods were also described. The results obtained with the screening tests and those obtained with the quantitative methods correspond well with each other. Using a combination of these methods, an effective control of residues of chloramphenicol can be performed in milk from the 1 μg/kg level and in swine tissues from the 10 μg/kg level.

INTRODUCTION

For the inspection of food of animal origin for veterinary drugs, generally an analytical strategy is recommended using at least two different methods. This strategy comprises [1]: (i) screening with a first method optimized to prevent false-negative results, with a high sample throughput, an acceptable percentage of false-positive results and low cost; and (ii) confirmation with an independent second method optimized to prevent false-positive results. In general, the second method will have a low throughput and high cost, and also give quantitative information.

A limited number of false-positive results is acceptable in a screening procedure. However, a low percentage of false-positive results can reduce the cost considerably, as all samples that show positive in the screening test must be submitted to a more expensive confirmatory method.

A confirmatory procedure is focused on the prevention of false-positive results. The method must be able to distinguish the analyte from all known interfering compounds in the matrix. De Ruig *et al.* [2] have suggested the applica-

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tion of certain criteria for the validation of qualitative analytical methods, which have to be fulfilled in the detection of the method. These criteria are already incorporated in a European Communities Commission Decision [3].

For the screening and determination of chloramphenicol (CAP) residues in food of animal origin at the low μg/kg level, different methods are available now (refs. 4 and 5 and references cited therein).

For screening purposes, enzyme-linked immunosorbent assays (ELISAs) are very suitable. In this study the following ELISAs were under investigation: (i) a commercially available immunochemical card test procedure, formerly called Quick-card and now called La Carte test [6,7], and (ii) a competitive monoclonal antibody-based streptavidin-biotin ELISA [8,9].

For quantitation of CAP two high-performance liquid chromatographic (HPLC) procedures, using both a solid-phase extraction (SPE) [10] and an antibody-mediated clean-up (AMC) [4,5] as a sample pretreatment were developed by us. The SPE procedure, using a silica SPE column, was originally developed for the determination of CAP in swine tissues [10] and somewhat modified for the determination of CAP in milk (see Experimental). The AMC procedure is based on a very specific clean-up and concentration of CAP from aqueous solutions (*i.e.*, aqueous meat extracts and defatted milk) using immobilized monoclonal antibodies directed against CAP. The AMC procedure was originally described for the determination of CAP in swine muscle tissues [4]. Later, this procedure was applied to milk and modified with respect to the support and the elution procedure for reasons of reuse of the immunoaffinity columns [5]. In this paper, the latter modifications were also applied for the determination of CAP in swine tissues. Moreover, in this study an immunoaffinity column with a greater bed volume was introduced to increase the column capacity (see Experimental).

This paper reports a comparative study using the above-mentioned screening and HPLC methods. For this purpose milk and swine tissue samples from CAP-treated animals were used.

EXPERIMENTAL

Reagents and chemicals

The reagents and chemicals used for the ELISA, the AMC and the SPE procedure were described earlier [5,8,10].

The monoclonal antibodies were prepared as described earlier [11]. These antibodies were biotinylated by Boehringer Mannheim (Tutzing/Obb, Germany) for use in the ELISA [8]. For use in the AMC procedure, the monoclonal antibodies were purified by ammonium sulphate precipitation and thereafter covalently bound to carbonyldiimidazole-activated trisacryl GF-2000 (Pierce, Rockford, IL, U.S.A.) as described earlier [5].

The immunochemical test kit, "La Carte test", was from Environmental Diagnostics (Burlington, DE, U.S.A.). The reagents and chemicals used for the appli-

cation of this test in milk are described by Nouws *et al.* [6] and for application in meat by Aerts *et al.* [7].

CAP was from Sigma (St. Louis, MO, U.S.A.). A CAP standard solution was prepared by dissolving 25 mg of CAP in 10 ml of methanol. Working standards for HPLC were prepared in the range 5–2500 ng/ml. Spiking solutions containing 0.10, 1.00, 5.00, 10.00 and 25.00 μg/ml CAP were prepared by diluting the standard solution in methanol. CAP used for the treatment of animals was a 50% CAP formulation from Alfasan (Woerden, The Netherlands).

The mobile phase solvent for HPLC was acetonitrile–0.01 *M* sodium acetate buffer, pH 5.4 (1:3, v/v).

Apparatus

The instruments used for the ELISA procedure were described earlier [8,9]. For the immunochemical card test procedure, the instruments are described by Nouws *et al.* [6] and Aerts *et al.* [7]. The instruments used for the AMC and the SPE procedure including the HPLC system were described earlier [5,10]. The detectors used in this study, however, were a 783A programmable absorbance detector (Applied Biosystems, Foster City, CA, U.S.A.) operated at 280 nm and a LC-235 diode-array detector (Perkin-Elmer, Pomona, CA, U.S.A.).

Samples

Swine tissues. To obtain real samples a swine (Great Yorkshire, weight 85 kg) was given a single intramuscular injection in the neck of 60 mg of CAP per kilogram body weight. The animal was slaughtered 64 h after administration.

The carcass was dissected by the Dutch method into different pieces of muscles (lean and streaky), fatty tissues and organs [12]. Before analysis, visible fat and connective tissue were removed from the pieces of lean meat as far as possible. The samples were submitted in duplicate to both the AMC procedure and the SPE procedure. The mean values were corrected for recovery (50 μg/kg spiking level, using the corresponding tissue of an untreated swine).

The "boston butt frontside" was "diluted" with the corresponding tissue of an untreated swine till the content was lower than the highest spiking level (*i.e.*, 1:70).

The untreated swine (Great Yorkshire, weight 96 kg) was slaughtered and dissected as described for the CAP-treated swine.

Milk. To obtain real samples, two cows (A: 665 kg; B: 610 kg) were each given a single intramuscular injection in the neck of 30 mg of CAP per kilogram of body weight. Milk samples were collected at each milking time for five days after drug administration.

The milk samples collected 15 and 23 h after CAP administration were diluted 1:10 and 1:5, respectively, with blank milk (*i.e.*, milk collected before the administration of CAP) for the AMC and SPE procedure.

All samples were submitted in duplicate to both the AMC procedure and the

SPE procedure. The mean values were corrected for recovery (50 μg/kg spiking level).

Milk samples collected before administration of the drug were used for spiking studies.

ELISA procedure

Sample preparation. The sample preparation for swine tissue was performed as described earlier [8]. The swine tissue samples were extracted with demineralized water and filtered. After addition of a phosphate-buffered saline (PBS) solution, the sample solutions ($n = 4$) were submitted to the ELISA.

The sample preparation for milk was performed as described earlier [9]. After centrifugation and filtration the milk samples ($n = 4$) were submitted to the ELISA.

Both for the swine tissue sample solutions and the milk sample solutions, the corresponding "blank" sample solutions were prepared by treatment with an immobilized monoclonal antibody preparation as described earlier [8,9]. The corresponding "blank" sample solutions ($n = 4$) were also submitted to the ELISA.

ELISA. The ELISA was performed as described earlier [8,9]. Wells of a microtitre plate, coated with a bovine serum albumin–CAP conjugate, were incubated with an aliquot of a sample solution together with an aliquot of a biotinylated monoclonal antibody solution. After this competition phase, the wells were successively incubated with a streptavidin–peroxidase conjugate solution and a substrate solution [2,2′-azino-di-(3-ethylbenzthiazoline-6-sulphonate)]. Between the different incubations the wells of the microtitre plate were washed using a microplate washer. After incubination with the substrate solution, the absorbance values were measured at 405 nm using an ELISA plate reader.

The result of the screening is considered to be positive if the mean absorbance of the sample solution is significantly lower than the mean absorbance of the corresponding blank, *i.e.*, a sample solution treated with immunoaffinity gel (normal Student *t*-test, one-tailed, $p < 0.05$) or if the absorbance of the sample solution is below 0.3. The latter condition was introduced to avoid false-negative results for strongly positive samples. In case of a very high CAP content, the amount of immunoaffinity gel, which is added to prepare a corresponding blank is insufficient to extract CAP completely from the sample. This results in low absorbance values for both the sample and the immunoaffinity-treated sample.

Immunochemical card test procedure

Sample preparation. The swine tissue samples were prepared according to the procedure described by Aerts *et al.* [7]. Samples were extracted with phosphate buffer (pH 5.0) using a Stomacher. The extract was filtered through a folded filter. An aliquot of this filtrate was filtered through a 0.45-μm filter. This sample solution was submitted to the La Carte test.

The milk samples were prepared as described by Nouws *et al.* [6]. The samples

were deproteinated with trichloroacetic acid. After addition of phosphate buffer to the deproteinized solution, the sample solution was submitted to the La Carte test.

La Carte test. The La Carte test procedure was performed according to the instructions for use included in each test kit. Each card contains two wells, *i.e.*, a control well and a sample well, with immobilized polyclonal antibodies directed against CAP. After addition of a blank control solution to a well and the sample solution to another well, a CAP–enzyme conjugate, a buffer solution and a substrate solution were successively added to both wells. The test is valid if there is colour formation in the control well within 5–15 min after addition of the substrate solution. If colour formation is observed in the sample well within the same time, the sample is considered to be negative.

AMC procedure

Sample preparation. The samples of swine muscle tissue and fatty tissue were prepared as described earlier [4]. The samples were extracted with demineralized water and filtered. After addition of a PBS solution to the filtrate, the sample solutions were submitted to AMC. For the organs a slight modification was introduced: the extract was centrifuged for 10 min at 10 000 *g* prior to filtration.

The milk samples were prepared as described earlier [5]. After centrifugation and filtration, the sample solution was submitted to AMC.

AMC was performed as described earlier [5], with the exception that a 2.5-ml bed volume of the immunoaffinity gel was used instead of a 0.5-ml bed volume. The sample solutions were pumped through these immunoaffinity columns by means of a peristaltic pump. The columns were washed with PBS. The antibody-bound CAP was eluted with 20 ml of a solution containing 0.2 *M* glycine and 0.5 *M* NaCl (pH 2.8). The eluate was extracted with ethyl acetate. The ethyl acetate fraction was evaporated to dryness, and the residue was dissolved in the mobile phase. The immunoaffinity columns were regenerated by washing the columns with PBS [5].

Chromatography. The samples were assayed under the HPLC conditions described earlier [10], except for the pH of the mobile phase and the detectors used. The programmable absorbance detector was used unless otherwise mentioned.

SPE procedure

Sample preparation. The swine tissue samples were prepared as described by Haagsma *et al.* [10]. The method comprises sonication-aided extraction with ethyl acetate, addition of hexane to the extract, and SPE using a small column packed with silica gel. Elution was performed with methanol.

For milk samples the following method was used. Approximately 10 g of homogenized milk was accurately weighed into a 50-ml polypropylene tube containing 0.4 g of citric acid monohydrate. CAP was extracted with 30 ml of ethyl acetate, using a shaking apparatus for 15 min. After centrifugation at 2300 *g* for

10 min, the organic layer was filtered through a filter paper (S&S 589.1). The extraction procedure was repeated with another 10 ml of ethyl acetate. The filter paper was washed with 10 ml of ethyl acetate. After addition of 60 ml of hexane to the total filtrate, the solution was filtered through filter paper (S&S 589.3). The total filtrate was submitted to SPE as described by Haagsma *et al.* [10] for swine muscle tissue.

Chromatography. The HPLC analysis was analogous to the procedure described for the AMC samples.

RESULTS AND DISCUSSION

Swine tissues

Spiking studies. Recovery experiments were carried out on ham muscle tissues for the modified AMC procedure at different CAP spiking levels (10, 50, 100 and 250 μg/kg). Each amount was added as eight replicates to the ground muscle tissue. The samples were submitted to HPLC analysis after AMC as described. The results are presented in Table I. The mean recoveries from spiked swine muscle tissues were 67 ± 4% (10–250 μg/kg). The loss of CAP could be completely attributed to incomplete aqueous extraction of the meat tissue. For the other procedures used in this study, spiking studies with CAP have been described elsewhere [7,8,10].

Real samples. All muscle tissues (lean and streaky), fatty tissues and organs of a CAP-treated swine were analysed for CAP according to the procedures described. Tissues of an untreated swine were also submitted to both the ELISA and the immunochemical card test procedure. The results are presented in Table II. Comparison of the results of the screening tests with the results of the quantitative methods demonstrates that all samples with a CAP content above the 10 μg/kg level, as determined with the HPLC procedures, also showed positive results with both the ELISA and the immunochemical card test procedure.

In general, the results obtained with the ELISA correspond well with the results of the immunochemical card test procedure (see Table II). Differences

TABLE I

RECOVERY OF CHLORAMPHENICOL FROM SPIKED SWINE MUSCLE TISSUES USING THE ANTIBODY-MEDIATED CLEAN-UP PROCEDURE

Added (μg/kg)	Recovery (%)	Standard deviation (%) ($n=8$)
10	70	3.1
50	67	2.8
100	65	3.6
250	66	2.6

TABLE II

SCREENING AND QUANTIFICATION OF RESIDUES OF CHLORAMPHENICOL IN TISSUES AND ORGANS OF A CAP-TREATED SWINE

The swine was given a singular intramuscular injection in the neck of 60 mg of CAP per kilogram of body weight. The animal was slaughtered 64 h after administration. AMC = antibody-mediated clean-up; SPE = solid-phase extraction; ELISA = enzyme-linked immunosorbent assay; N.A. = not applicable; N.D. = not detectable (< 1 μg/kg).

Tissue	Weight (g)	CAP content (μg/kg)		Result (+/−)	
		AMC	SPE	ELISA[a]	La Carte test[a]
Lean tissue					
Diaphragm[b]	183	48	44	+(−)	+(−)
Bottom round	1900	52	51	+(−)	+(−)
Eye of round	600	47	52	+(−)	+(−)
Fore shank	600	50	49	+(−)	+(−)
Hind shank	1300	44	46	+(−)	+(−)
Top round	2600	46	50	+(−)	+(−)
Knuckle + bottom butt loinside	1850	45	40	+(−)	+(−)
Sirloin	2400	45	49	+(−)	+(−)
Loin hip end	1900	39	44	+(−)	+(−)
Loin	2850	47	44	+(−)	+(−)
Tenderloin	650	53	50	+(−)	+(−)
Shoulder picnic	3910	51	51	+(−)	+(−)
Streaky tissue					
Boston butt frontside	2100	3616	3315	+(−)	+(−)
Boston butt loinside	1450	34	33	+(−)	+(−)
Belly	4680	22	22	+(−)	+(−)
Fatty tissue					
Ventral part of the belly	550	N.D.	N.D.	−(−)	+/−(+/−)
Ham fat	1100	2	2	−(−)	+/−(−)
Jaw	1500	17	19	+(−)	+(+/−)
Flare	785	N.D.	N.D.	−(−)	+/−(−)
Back fat	1500	3	4	−(−)	+(+/−)
Shoulder fat	400	14	13	+(−)	+(−)
Organs					
Tongue	240	11	9	+(−)	+(−)
Heart	220	18	16	+(−)	+(−)
Liver	1100	N.D.	N.A.	−(−)	N.A.
Kidney	183	23	30	+(−)	N.A.

[a] The results of ELISA and La Carte test performed on blank swine muscle tissues are given in brackets.
[b] The pillar of the diaphragm was included.

were observed only in some fatty tissues and organs. Screening of fatty tissues with the immunochemical card test procedure leads to some doubtful results (*i.e.*, colour formation that is less than that obtained in the control well) for the real

samples as well as the blank samples. Liver and kidney samples could not be screened with the immunochemical card test procedure owing to clogging of the filter paper. In the blank tissues no false-positive results could be observed in either the ELISA or the La Carte test.

The lowest detectable CAP level for both immunochemical tests was established. For this purpose two muscle tissues (fore shank and shoulder picnic) of the CAP-treated swine were "diluted" in different ratios with the corresponding tissue of the untreated swine. The results indicate that, for the ELISA procedure, the lowest detectable CAP level is between 3 and 6 μg/kg and, for the immunochemical card test procedure, it is between 6 and 12 μg/kg. The ELISA results correspond well with the results of the spiking studies described earlier [8]. The results obtained with the immunochemical card test procedure slightly differ from the 1–4 μg/kg limit of detection range reported by Aerts *et al.* [7]

The results obtained by the AMC and the SPE procedure correspond well with each other (see Table II). Except for the liver samples, good recoveries were obtained with both procedures. The CAP contents of the fatty tissues and organs are considerably lower than the CAP contents of the lean tissues. The variation of the CAP content in the different lean tissues is relatively small. These results correspond well with those described earlier [10].

The analysis of CAP in liver deserves some attention. The SPE procedure could not be used at all owing to matrix interferences in the HPLC analysis. These interferences were not observed when the AMC procedure was used. However, CAP could not be detected in the real liver sample. After spiking the blank liver tissues with CAP, very low recoveries (*ca.* 14%) were observed. On the contrary, good recoveries (*ca.* 95%) were obtained after spiking to blank liver extracts just before AMC. These results are in accordance with those found by Parker and Shaw [13]. They also demonstrated that CAP recoveries from spiked bovine liver were poor, owing to rapid *in vitro* metabolism of CAP to CAP-glucuronide. However, in our experiments, incubation of spiked liver tissue extracts with β-glucuronidase before the AMC did not improve the recovery.

The limit of detection of the CAP standard in the HPLC system using the programmable absorbance detector was established at 0.18 ng (signal corresponding to three times the noise level), which is about three times lower than the detection limit described earlier [5]. As a result, quantitation could be performed at the 1 μg/kg level for both HPLC procedures. The HPLC was also performed using full-spectrum diode-array detection. CAP could be identified in samples with a CAP content above the 8 μg/kg level. The CAP spectra obtained from real samples are completely identical with that of standard CAP, including the wavelength of maximum absorption. A chromatogram and spectrum of the CAP peak of a real swine muscle tissue (top round) obtained with the diode array detector is presented in Fig. 1, for both the AMC and SPE procedure.

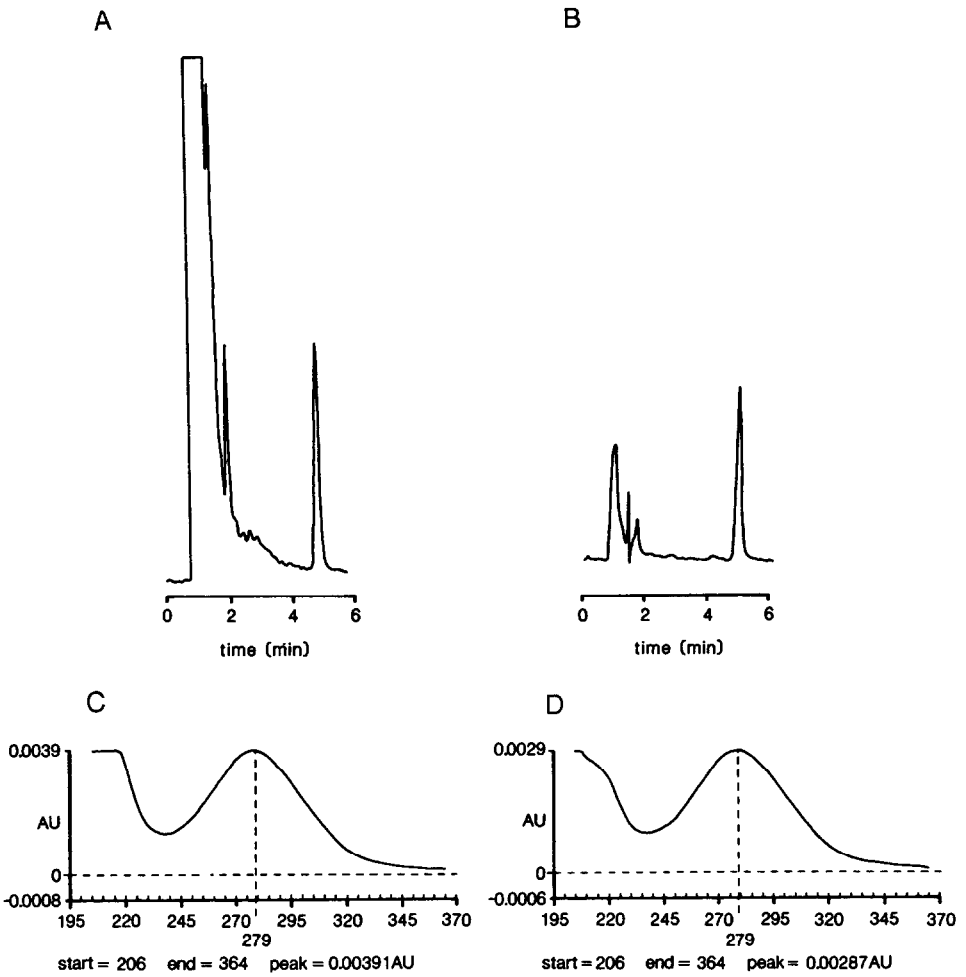

Fig. 1. Chromatograms of extracts of real swine muscle (top round; 48 μg/kg) and spectra of the CAP peak. The chromatograms were recorded at 280 nm (absorbance range settings, 0.020 a.u.f.s). (A) and (C) SPE procedure; (B) and (D) AMC procedure.

Milk

Spiking studies. Recovery experiments were carried out for the modified AMC and SPE procedures at the 1, 10, 100 and 250 μg/kg levels with spiked milk samples ($n = 6$). The results are presented in Table III. The mean recoveries from spiked milk samples were 67 ± 5% (1–250 μg/kg) for the AMC procedure and 68 ± 5% (1–250 μg/kg) for the SPE procedure. Both procedures were able to quantitate CAP at the 1 μg/kg level. The AMC procedure described earlier [5] showed recoveries of 82–100%. These differences might be explained by the difference in type of milk (raw tank milk samples and pasteurized full-cream milk samples, respectively). The recoveries obtained with the SPE procedure are somewhat

TABLE III

RECOVERY OF CHLORAMPHENICOL FROM SPIKED MILK USING THE AMC AND SPE PROCEDURES

Amount added (μg/kg)	AMC		SPE	
	Recovery (%)	Standard deviation ($n=6$)	Recovery (%)	Standard deviation ($n=6$)
1	61	2.4	68	5.3
10	68	1.7	65	5.5
100	72	5.2	70	5.5
250	66	3.1	70	4.4

lower (± 10%) than those described earlier for the determination of CAP in swine muscle tissues [10].

Real samples. Milk samples from two CAP-treated cows were collected at each milking time for five days and analysed for CAP according to the procedures described. The results are presented in Table IV. Comparison of the results of the screening tests with the results of the quantitative procedures demonstrates that all samples with a CAP content equal or above the 1 μg/kg level, as determined with the HPLC procedures, also showed positive results with the ELISA procedure. The immunochemical card test procedure, however, could detect CAP in milk only at or above the 5 μg/kg level. Recent investigations described by Aerts *et al.* [7] showed that the limit of detection of this direct immunochemical screening in milk can be lowered to the 1 μg/kg level by applying 200 μl of the sample solution to the La Carte test instead of 50 μl. We later applied this slightly modified procedure to real milk samples. However, some doubtful results were obtained in the range 1–2 μg/kg.

The results obtained by the AMC and SPE procedures correspond well with each other (see Table IV). The correlation between the results obtained with the AMC (x value) and SPE procedure (y value) is given by the lines $y = 3.49 + 1.00x$ (for cow A) and $y = 6.65 + 0.98x$ (for cow B), both with a coefficient of correlation of 0.9997.

The HPLC procedures were also performed using full-spectrum diode-array detection. CAP could be identified in samples with a CAP content above the 8 μg/kg level. The CAP spectra obtained from real samples were completely identical with that of standard CAP, including the wavelength of maximum absorption. The chromatograms and spectra were similar to those presented for swine tissues.

Reliability of the analytical procedures

The ELISA procedure used in the comparative study was developed for screening purposes at the 10 μg/kg level in swine tissue and at the 1 μg/kg level in

TABLE IV

SCREENING AND QUANTIFICATION OF RESIDUES OF CHLORAMPHENICOL IN MILK OF TWO TREATED COWS

For CAP treatment, see text.

Time after injection (h)	Cow	CAP content (μg/kg)		Result +(−)	
		AMC	SPE	ELISA	La Carte test
0	A	<1	<1	−	−
	B	<1	<1	−	−
15	A	2015	1998	+	+
	B	2447	2389	+	+
23	A	678	717	+	+
	B	686	730	+	+
39	A	62	61	+	+
	B	61	62	+	+
47	A	27	23	+	+
	B	25	24	+	+
63	A	6	6	+	+
	B	7	7	+	+
71	A	2	3	+	+/−
	B	5	4	+	+/−
87	A	2	2	+	+/−
	B	2	2	+	+/−
95	A	1	2	+	+/−
	B	1	1	+	+/−
111	A	<1	<1	+	−
	B	<1	<1	+	−
119	A	<1	<1	−	−
	B	<1	<1	−	−

milk. All samples with a CAP content above these levels gave positive results in the ELISA (no false negatives). The immunochemical card test procedure showed similar results, with the exception of the doubtful results at lower CAP levels in milk. This will not be a problem if the modified procedure is applied [7].

Neither the ELISA nor the immunochemical card test produced any false positives in the comparative study. Moreover, the ELISA was used to screen large numbers of samples from practical situations (results not shown). No false-positive results were observed here either. Apart from this, a high throughput of samples can be attained with both the ELISA and immunochemical card test procedure. Compared with the immunochemical card test, the ELISA procedure is easier to automate.

Recently, de Ruig *et al.* [2] introduced criteria for the detection of analytes in test samples. For liquid chromatography the nearest peak maximum in the chromatogram should be separated from the designated analyte peak by at least one full width at half the maximum height. This criterion was fulfilled for both proce-

dures: no interfering peaks were observed in the chromatogram. In particular, very clean chromatograms were obtained with the AMC procedure. For identification, additional co-chromatography is mandatory [2]. Only the peak presumed to be due to CAP was intensified, and the retention times were completely identical.

Apart from the prevention of false-positive results, the HPLC methods have also been developed for quantitative purposes. The quality of quantitative analytical procedures can be expressed in terms of the accuracy, comprising both systematic errors and random errors, expressed as trueness and precision, respectively [14]. The good correspondence of the results obtained with the two completely different sample pretreatment methods used in the comparative study (*i.e.*, AMC and SPE procedures) is an indication that the contribution of the systematic errors to the measured value is small. The contribution of random errors is reflected by the standard deviation. For both methods a low standard deviation was obtained for the repeatability, as established by analysis of spiked tissues.

In a complete analytical strategy, the screening method and the method used for confirmation/identification must be independent [1]. However, the AMC procedure is based on an immunochemical technique and is therefore not totally different from the ELISA and the immunochemical card test procedure. On the contrary, the SPE procedure is totally independent of these two screening methods. Therefore, in this respect, the SPE procedure is to be preferred for confirmation purposes after screening with the ELISA or immunochemical card test procedure.

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