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Design of a sensitive fluorescent polarization immunoassay for rapid screening of milk for cephalexin

Natalia V. Beloglazova[1] · Sergei A. Eremin[2]

Abstract In this paper we describe the development of a sensitive, fast, and easily performed fluorescence polarization immunoassay for determination of cephalexin in milk. The experimental work was performed to increase sensitivity and specificity. Therefore, the structures of the tracers were varied by synthesis of both cephalexin (CEX) and cephalotin (CET) conjugates with a variety of fluorescent labels. Two rabbit antisera containing antibodies against cephalexin and cephalotin were tested in homologous and heterologous combinations with the tracers. For every working antibody–tracer combination, the analytical conditions and cross-reactivity for structural analogues—cephalosporins and other antibiotics that could also be present in milk—were determined. It was found that the highest sensitivity was achieved by use of the homologous pair CET–EDF–anti-CET antibody (limit of detection (LOD) 0.4 µg kg^{-1} for standard solutions prepared in buffer), but this combination was not appropriate because of high cross-reactivity with CET. For subsequent experiments, therefore, CEX–EDF–anti-CEX antibody were chosen (LOD 0.8 µg kg^{-1} for standard solutions prepared in buffer). Part of this manuscript is devoted to the variation of precipitation agents for pretreatment of milk before analysis; milk is an extremely complicated matrix. The optimum protein precipitation agent was methanol. This technique for cephalexin determination was characterized by a limit of detection of 1 µg kg^{-1}. The method was validated by using naturally contaminated and spiked milk samples. The results obtained corresponded very well with those obtained by HPLC, which was used as confirmation method.

Keywords Fluorescence polarization immunoassay · Cephalexin · Cephalosporins · Milk · Affinity constants · Antibiotics

Introduction

Resistance of human pathogens to antibiotics is a serious and fast-growing threat to contemporary medicine [1] which is regarded as a major public health concern of the 21st century. According to a World Health Organization (WHO) report " … antibiotic resistance—when bacteria change so antibiotics no longer work in people who need them to treat infections—is now a major threat to public health" [2]. For example, the report reveals high levels of resistance of *K. pneumoniae* to third-generation cephalosporins throughout the WHO European Region. Cephalosporins are antibiotics used for therapy and prevention of infectious diseases regularly affecting livestock animals. Although the extensive use of antibiotics in human medicine is the major cause of resistance to antibiotics, their unreasonable application in farming also has a huge effect. Cephalosporins are now administered to animals not only for preventive and prophylactic purposes—they are also used illegally at subtherapeutic levels to increase feed efficiency and to promote growth in food-producing animals [3]. As a result of either this illegal use or non-compliance with treatment protocols, antibiotics residues being found at different

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concentrations in products of animal origin, for example meat and milk. Antibiotics residues in foodstuff not only provoke different allergic reactions among hypersensitive individuals, affect the bacterial flora of the human intestinal tract [4], and spread drug-resistant microorganisms [5], they also increase the resistance of pathogenic bacteria to antibiotics [6]. Development of rapid, sensitive techniques for determination of cephalosporins in milk is, therefore, urgently needed.

Cephalexin ((6R,7R)-7-{[(2R)-2-amino-2-phenylacetyl]amino}-3-methyl-8-oxo-5-thia-1-azabicyclo [4.2.0]oct-2-ene-2-carboxylic acid; CEX), a first-generation cephalosporin, is a semi-synthetic antibiotic. CEX is a broad-spectrum drug effective against Gram-positive and Gram-negative bacteria. CEX is used to treat bronchitis, tonsillitis, infections of the ear, skin, and urinary tract, and even heart disease, and CEX one of the most-used drugs worldwide (under different brand names) [7]. CEX is one of eight cephalosporins approved for use in the European Union; its maximum residue level (MRL) in milk is 100 μg kg^{-1} [8].

Many chromatographic techniques are used for analysis of CEX in milk [9–12]. Capillary electrophoresis (CE) has been used in combination with, mainly, diode-array detection (DAD) [13–15]. Despite good specificity and sensitivity these methods require use of expensive and complex equipment and high-cost and time-consuming sample-preparation steps. They cannot, therefore, be used for high-throughput screening, so there is a growing demand for reliable, sensitive, and, at the same time, easy-to-operate and low-cost techniques for rapid screening of large numbers of samples.

For preliminary screening of cephalosporins in milk, rapid microbiological tests (Delvotest, Copan Milk, Eclipse 100) are commercially available and often used [16]. Extensive incubation time and poor sensitivity has, however, resulted in progressive replacement of microbiological kits by rapid immunochemical tests (Charm Rosa, SNAP MRL, Penzym, BetaXpress Milk) because of their speed and convenience [17, 18]. These immunochemical tests are mostly qualitative or semi-qualitative, because of lack of specificity, which can lead to incorrect results. A variety of instrumental immunochemical techniques, for example ELISA [19–21], an SPR-based immunosensor [22], and a laser-induced fluorescence-based immunoassay [23], have already been developed for analysis of CEX in milk. Sensitivity and specificity are good but they are quite time-consuming.

Fluorescence polarization immunoassay (FPIA) is a competitive method characterized by good sensitivity and specificity, easy operation, and high throughput. Portable FPIA readers enable on-site measurement, which facilitates rapid screening of samples. A fluorescence polarization technique for determination of CEX in milk was recently reported [24]. However, the authors did not study the effect of tracer structure on sensitivity and their work lacked an extensive study of possible sample-pretreatment procedures. In this manuscript,

a highly sensitive FPIA technique for determination of cephalexin in milk is described. To the best of our knowledge this is the first paper to describe design of tracer structures, variation of homologous and heterologous immunoreagents, and determination of the optimum sample-pretreatment procedure in the development of a sensitive FPIA technique for determination of cephalexin.

Material and methods

Reagents and materials

Fluorescein isothiocyanate (FITC) isomer I, fluoresceinamine (AF) isomer I, ethylenediamine dihydrochloride, N-hydroxysuccinimide (NHS), N,N-dicyclohexylcarbodiimide (DCC), triethylamine (TEA), trichloroacetic acid (TCA), cephalexin hydrate (CEX), cephalotin sodium salt (CET), gentamicin, ampicillin, streptomycin, chloramphenicol, and sodium azide were purchased from Sigma–Aldrich (Bornem, Belgium). 4-(Aminomethyl)fluorescein (AMF) hydrochloride was purchased from Life Sciences (Ghent, Belgium). Rabbit antisera containing |the polyclonal antibody against CEX and the polyclonal antibody against CET were kindly provided by Professor Ch. Xu (School of Food science and Technology, Southern Yangtze University, WuXi, China). Antiserum against CEX was obtained as described by Xie et al. [25]. The antibody was characterized by 96 % cross reactivity toward cefadroxil and ~30–40 % cross-reactivity toward other cephalosporins (including 33.1 % cross-reactivity toward CET). All other chemicals and solvents were of analytical grade. Ultrapure water was used throughout. Borate buffer (BB, 2.5 mmol L^{-1}, pH ~7.5, containing 1 % (w/v) sodium azide as preservative) was used as assay buffer. Standard solutions of the antibiotics were prepared by diluting the reference stock solution (1 mg mL^{-1} in PBS) in the range 0.001–100 ng mL^{-1}).

All fluorescence polarization measurements for FPIA were performed with a TDx polarization fluorimeter (Abbott Laboratories, US) in PhotoCheck mode.

Synthesis of ethylenediamine fluoresceinthiocarbamyl

Ethylenediamine fluoresceinthiocarbamyl (EDF) was synthesized by the modified technique described by Eremin et al. [26]. Ethylenediamine dihydrochloride (200 mg, ~1.5 mmol) dissolved in a mixture of methanol (5 mL) and TEA (500 μL) was added dropwise to FITC solution (117 mg, ~300 μmol) in methanol (10 mL) containing 100 μL TEA. The solution was mixed for 1 h at RT then the bright orange pellet was isolated by filtration and dried.

Synthesis of fluorescent labeled conjugates (tracers)

FITC, AF, AMF and EDF were used as labels for synthesis of tracers. The structures of the prepared conjugates are presented in Fig. 1.

1. To synthesize tracers labeled with EDF, AF, and AMF, the carboxyl group was activated by use of NHS and DCC. NHS (12.3 mg, approx. 107 μmol) was dissolved in 2.7 mL DMF, 21 mg DCC (~102 μmol) was dissolved in 2.5 mL DMF, and 250 μL of each solution was added dropwise to 3.4 mg (~10 μmol) CEX or 3.9 mg (~10 μmol) CET dissolved in DMF. The reaction mixture was stirred overnight at RT. The precipitate formed was removed by centrifugation (10 min, 10,000 g). EDF (~4.5 mg), AF (~3.6 mg), or AMF (~3.6 mg) was added to the supernatant and the reaction mixture was stirred for 2 h at RT in the dark, followed by overnight incubation at 4 °C.
2. CEX–FITC was synthesized by dissolving the antibiotic (~3.4 mg, ~10 μmol) and the label (4 mg, ~10 μmol) in methanol (1 mL) containing TEA (50 μL) and incubation overnight at 4 °C with constant stirring.

To remove impurities and starting reagents the synthesized tracers were separated and purified by the thin-layer chromatography (TLC) on Silufol (Czech Republic) chromatographic plates with a silica gel layer thickness of 0.25 cm; methanol–chloroform 1:4 (*v*/*v*) was used as mobile phase. The main yellow bands, which were luminescent in UV light (λ= 365 nm), were collected from the chromatographic plate and extracted with methanol. The tracers were kept at 4 °C.

Fluorescence polarization immunoassay

Optimization of the analytical procedure started with determination of the optimum concentrations of the immunoreagents. These tracer working concentrations required a total final fluorescence intensity ten times higher than the background signal The working concentrations of antisera were determined from a plot of antiserum dilution as a function of fluorescence polarization. Dilutions in the range 1:100 to 1:102400 were performed with borate buffer solution (the final volume was 500 μL). Tracer solution (500 μL) at the optimum concentration was added to all the antiserum dilutions and the fluorescence polarization (FP) was measured. The dilution curve was plotted on a semi logarithmic scale, and the optimum antisera dilutions corresponded to 70 % of the tracer's binding response to the antibodies.

To construct an FPIA calibration curve, standard solutions of the target antibiotics (CEX and CET) were prepared in BB. Each standard solution (50 μL), working tracer solution (500 μL), and antibody solution (500 μL) were added, the
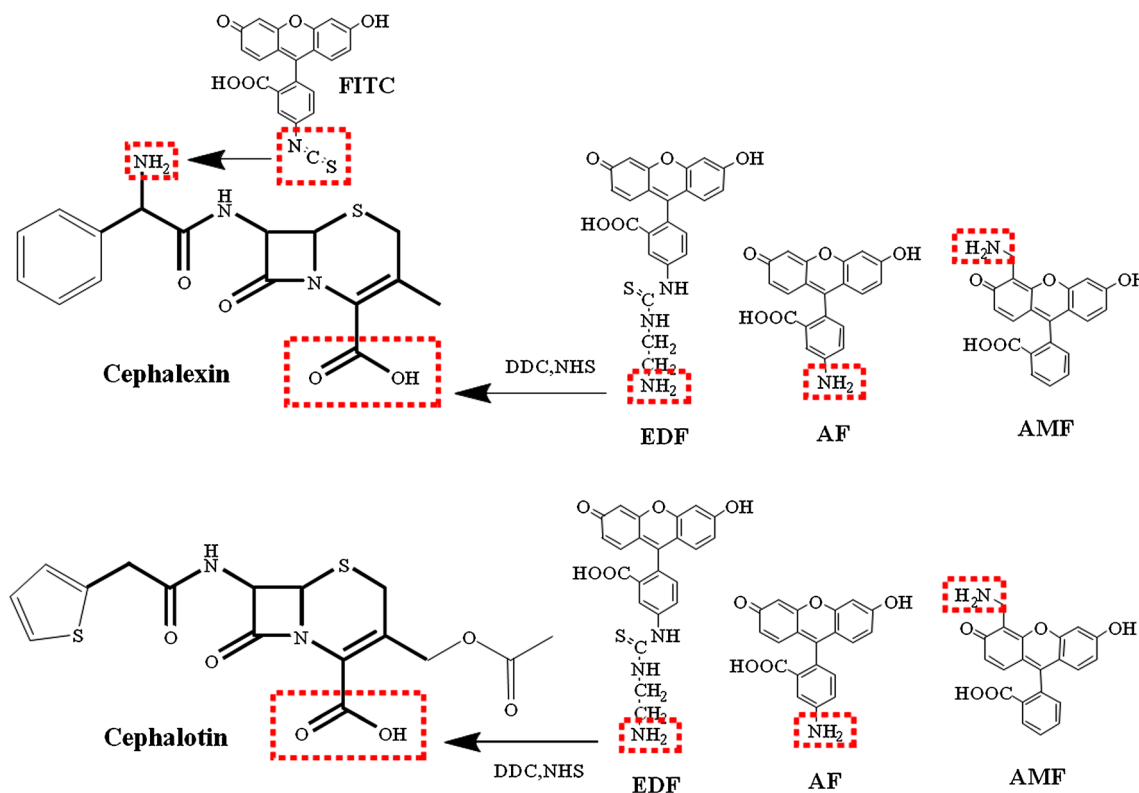
Fig. 1 Structures of the synthesized tracers

mixtures were stirred and the fluorescence polarization was measured and expressed in "milli-polarization" units (mP).

Analytical performance of FPIA

The relative unit mP/mP_{max}, where mP_{max} was the maximum FP value of the calibration curve and mP was a measured value, was proposed for normalization of the FP value. Standard FPIA sigmoidal curves were plotted on a semi logarithmic scale with relative FP values on the y axis, and the x axis provided the logarithm of analyte concentration. These curves were described by a Rodbard four-term function:

$$y = (A-D)/\left[1 + (x/C)^b\right] + D$$

where A is the maximum FP value, D the minimum FP value, b the slope of the curve in the IC_{50} plot, and C the IC_{50} concentration of the analyte. The IC_{50} value, the dynamic range, and the limit of detection (LOD), were evaluated. The LOD was defined as the concentration of standard solution that furnished an analytical signal three times the signal-to-noise ratio:

$$mP_{min} = mP_0 - 3S$$

where mP_{min} is the fluorescence polarization corresponding to the limit of detection, mP_0 is the average value of the fluorescence polarization, based on 20 measurements of the zero standard solution of the antibiotic, and S is the standard deviation for 20 measurements of the analytical signals.

The IC_{50} value, the amount resulting in 50 % binding inhibition in the fluorescence polarization assay, was used as the sensitivity of the assay. The dynamic range was defined as the binding inhibition in the fluorescence polarization assay with a value between 20 and 80 %. The specificity of the FPIA was estimated by use of the cross reactivity calculation:

$$CR\ \% = \frac{IC_{50}(CEX)}{IC_{50}(analyte)} \cdot 100\%$$

Sample preparation

Whole milk and skimmed milk were purchased locally (China, Russia). For preparation of milk samples, 1 mL methanol was added to 500 μL milk to minimize interference from the milk matrix. The mixture was stirred for 10 min and then centrifuged for 6 min at 10, 000 rpm, and the precipitate was removed. The supernatant was used for subsequent analysis.

Result and discussion

Choice of immunoreagents

Structural design of the tracer is an important step in the development of FPIA for detection of low-molecular-weight-analytes. Therefore, the fluorescence-labeled conjugates of cephalotin (CET–EDF, CET–AF, CET–AFM), an antibiotic structurally similar to cephalexin, were designed with the CEX-based tracers (CEX–FITC, CEX–EDF, CEX–AF, CEX–AFM). The lowest possible tracer concentration that enables reliable detection without any effect of competing compounds should be chosen to obtain the highest possible sensitivity. Binding of all the synthesized tracers with two different antisera, against CET and CEX, were tested. For CEX–FITC, CEX–AF, CEX–AFM, CET–AF, CET–AFM binding was not sufficient. The best coupling was between CEX–EDF and CET–EDF in homologous and heterologous pairs (Fig. SI1). The affinity constants were calculated to characterize the observed binding behavior. Because the antibodies used were polyclonal, the affinity constants were estimated for high and low-affinity fractions. This approach enabled evaluation of the binding and comparison of the tracer–antibody immunoreagent pairs. As is apparent from the results listed in Table 1, high values were obtained for all the affinity constants. Better binding was observed for homologous pairs of the immunoreagents, especially by CET–EDF–anti-CET antibody, than for heterologous pairs.

Fluorescence polarization immunoassay

Calibration curves for CEX determination using different pairs of immunoreagents are plotted in Fig. 2a.

Sensitivity

Analytical characteristics of the developing FPIA are presented in Table 2. The best results (the lower IC_{50} and LOD values) were obtained by use of CET–EDF–anti-CET-antibody. The analytical characteristics obtained were better than

Table 1 Affinity constants of the anti-cephalosporins antibodies ($n=5$)

Immunoreagent	Affinity constant (mol^{-1} L)	
	High-affinity fraction	Low-affinity fraction
CEX–EDF–anti-CEX antibody	$2.6\pm0.2\times10^9$	$2.5\pm0.5\times10^7$
CEX–EDF–anti-CET antibody	$7.4\pm0.4\times10^8$	$1.8\pm0.2\times10^6$
CET–EDF–anti-CEX antibody	$3.4\pm0.3\times10^8$	$1.8\pm0.2\times10^6$
CET–EDF–anti-CET antibody	$1.3\pm0.2\times10^9$	$1.6\pm0.3\times10^7$
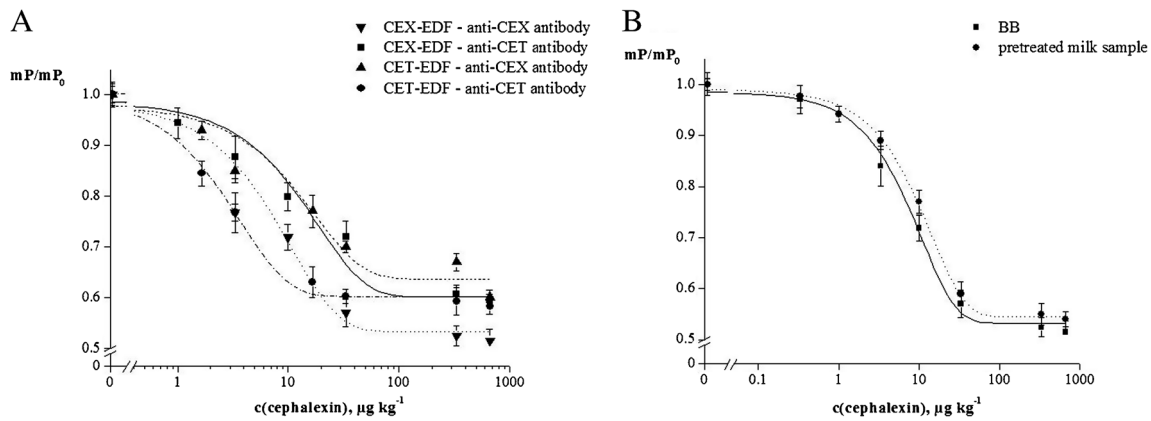
Fig. 2 **a** Calibration curves for determination of CEX by use of two different antisera (anti-CEX and anti-CET), two different tracers (CEX–EDF and CET–EDF), standard solutions prepared in the buffer. **b** Calibration curves for determination of CEX in buffer solution and blank milk extract ($n=5$)

those described by Zhang et al. [24] (LOD 1.8 ng mL^{-1}; IC$_{50}$ 10.8 ng mL^{-1}), indicating that our technique was more sensitive. Taking into account the pretreatment procedures, the ELISA based on this anti-CEX antiserum was more sensitive (LOD 0.2 ng mL^{-1}; IC$_{50}$ 1.5 ng mL^{-1}) [25] but the FPIA technique is much faster (10 samples could be analyzed within 10 min) and easier. The LODs obtained were also lower than the established MRL for CEX in milk.

Specificity

The specificity of FPIA for CEX determination was assessed by calculation of the cross-reactivity with a large number of cephalosporins (cephalotin, cefadroxil, cefotaxine, cefazolin, ceftiofur, and cefapirin) effective against a broad range of Gram-positive and Gram-negative bacteria and commonly used for treatment of clinical mastitis among lactating cows caused by *Escherichia coli* and *Streptococcus* and *Staphylococcus* species, and other antibiotics also widely used in veterinary medicine which could be present in milk, namely ampicillin, gentamicin, chloramphenicol, and streptomycin (Table 3). Results obtained by use of the developed FPIA method for the anti-CEX antiserum correlated well with the cross-reactivity values determined by ELISA [25]. Because both polyclonal antibodies used had high cross-reactivity with

other cephalosporins, because of their highly similar structures (cross-reactivity with the other above-mentioned antibiotics was negligible), the developed FPIA could be used for multi-residue detection of cepfalosporins as a preliminary screening method. Although use of the anti-CET antibody resulted in better sensitivity, its cross-reactivity with CET was more than 200 %. In this case application of the anti-CEX antibody looked more reasonable. In homologous combination the with tracer, the sensitivity was still very good (IC$_{50}$~7.5 µg kg^{-1}) and enabled multiple dilution of milk samples to reduce matrix effects. Therefore, all subsequent experiments were performed with CEX–EDF–anti-CEX antibody reagents.

Pretreatment of milk samples

Milk is a very complex matrix and can affect the performance of the analysis. Choice of optimum sample pretreatment is an

Table 2 Analytical performance characteristics for determination of cephalexin in standard solutions by use of the FPIA with different immunoreagents ($n=5$)

Immunoreagent	LOD (µg kg^{-1})	Linear range (µg kg^{-1})	IC$_{50}$ (µg kg^{-1})
CEX–EDF–anti-CEX antibody	0.8	2.4–20	7.5±0.8
CEX–EDF–anti-CEP antibody	1.3	3.5–30	12±0.7
CEP-EDF–anti-CEX antibody	0.9	3.3–36	12±1
CEP-EDF–anti-CEP antibody	0.4	0.8–11	2.6±0.4

Table 3 Cross-reactivity of the anti-CEX polyclonal antibody (checked in combination with CEX–EDF) ($n=5$)

Compound	Cross-reactivity (%)			
	anti-CEX antibody		anti-CET antibody	
	CEX–EDF	CET–EDF	CEX–EDF	CET–EDF
Cephalexin	100	100	100	100
Cephalotin	32.3	39.1	201	225
Cefadroxil	91.7	94.3	79.3	82.1
Cefotaxine	28.4	33.1	20.7	22.4
Cefazolin	47.1	45.6	36.5	41.8
Ceftiofur	38.5	37.4	22.4	26.7
Cefapirin	41.4	42.2	32.1	37.3
Ampicillin	3.11	6.18	6.27	3.11
Gentamicin	<0.1	<0.1	<0.1	<0.1
Chloramphenicol	<0.1	<0.1	<0.1	<0.1
Streptomycin	<0.1	<0.1	<0.1	<0.1

important step in development of any analytical technique. It might be a simple defatting procedure, for example centrifugation, followed or not followed by heating for inactivation of endogenic alkaline phosphatase used in some techniques, for example the lateral-flow test [21, 25], ELISA [20], flow-through amperometric immunoanalysis [27], and SPR-based biosensing [28]. For chromatographic determination of cephalosporins, milk samples have been treated with acetonitrile [11, 12, 29], hexane [30], or acetic acid [31] for deproteination, then by liquid–liquid [30] or solid-phase [11, 12] extraction. For immunochemical screening of milk, samples were defatted by centrifugation [20, 25, 27]. A previously reported FPIA for cephalexin determination was based on use of 1.25 % TCA for protein precipitation [24]. In our work, together with 1 % TCA and the organic solvents methanol, ethanol, and acetonitrile, a saturated solution of ammonium sulfate was tested as precipitating agent. To compare interference from all these precipitation agents for each sample, unspiked and spiked (CEX 3 µg kg^{-1}) portions were treated with each precipitation agent. The ammonium sulfate solution and TCA were added to samples in the ratio 1:1 (v/v) and the organic solvents were added in the ratio precipitation agent:milk 2:1 (v/v). All samples were stirred, centrifuged, and the supernatants were collected and analyzed by use of the FPIA.

As criterion for comparing the effectiveness of precipitation agents the relative decrease of the analytical signal (FP) after changing the cephalexin concentration from 0 to 3 µg kg^{-1}, i.e. $(\text{mP}_{0 \text{ µg kg-1}} - \text{mP}_{3 \text{ µg kg-1}})/\text{mP}_{0 \text{ µg kg-1}}$, expressed as a percentage, was selected. The results were compared with those for a standard solution for which twice-distilled water served as supernatant (Fig. 3). A low relative decrease was indicative of an effect of the milk matrix on the FPIA measurements, which prevented correct interpretation of results obtained. It should be mentioned that the effect of the pH of the milk on determination of cephalexin by FPIA
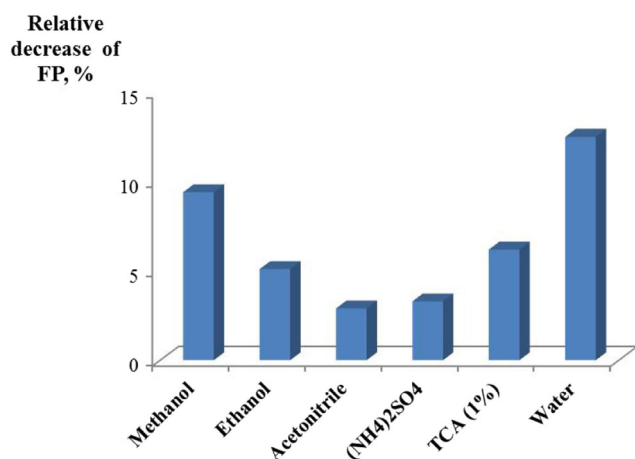
was negligible, because of the quite high buffering capacity of BB used as working buffer. On the basis of the experimental results it can be concluded that saturated ammonium sulfate and acetonitrile as precipitation agents only slightly reduced interference from the milk matrix. In contrast, methanol effectively eliminated the matrix effect: a high mP$_{0\text{ng mL}-1}$ value is indicative of effective removal of interfering proteins and insignificant matrix effect of this solution compared with distilled water. For correction for matrix effects a calibration curve was constructed by using CEX standard prepared in blank milk supernatant (as a blank sample sterilized milk was used, Fig. 2b). The change of the sensitivity of the assay was insignificant: LOD and IC$_{50}$ values were 1 and 9.5 µg kg^{-1}, respectively. Methanol did not remove the matrix effect completely, but sufficiently for our purposes: the LOD of the technique was much lower than the established MRL for CEX in milk.

All the results mentioned above testified that the developed FPIA technique could be used for screening real milk samples for CEX.

Analysis of artificially-spiked and naturally-contaminated milk samples

First, the applicability of the FPIA method was estimated by analysis of sterilized milk samples artificially spiked with CEX. Samples spiked with CEX at concentrations greater than the linear range of the FPIA were diluted with BB after the pretreatment procedure, before analysis. The correlation between amounts of antibiotic added and the concentrations found is given in Fig. 4a. The graph is indicative of good applicability of the technique for screening of milk samples.
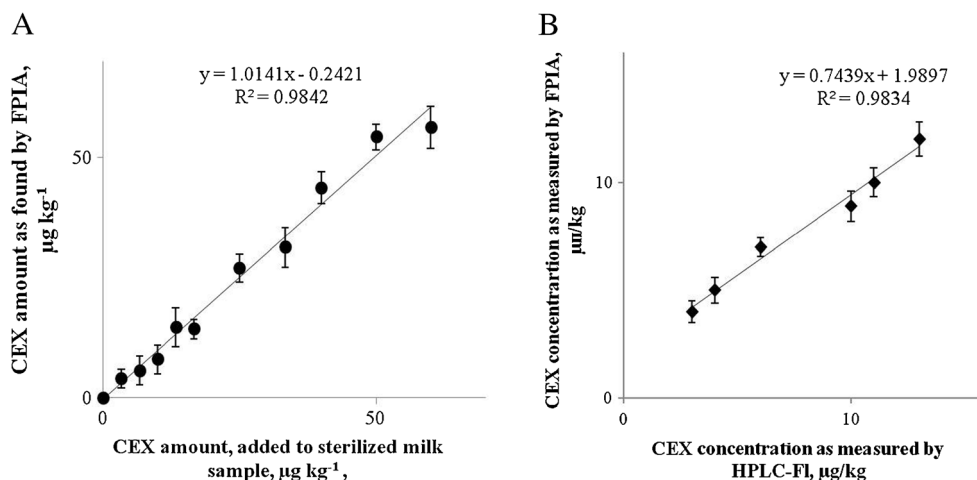
Thirteen naturally contaminated milk samples from Russian and Chinese manufacturers were analyzed in triplicate by use of this procedure. Samples which contained CEX at concentrations below the LOD of the technique were concentrated before analysis. CEX was present in six samples at concentrations above the LOD, in the range 3–13 µg kg^{-1}.

An HPLC method based on that described by Oliveira et al. [32] was used as confirmation technique. Good agreement was obtained for the samples which were found to be contaminated ($r^2 = 0.9834$, Fig. 4b).

Conclusion

A sensitive and rapid homogeneous FPIA for determination of cephalexin in milk has been developed and validated. Two different rabbit antisera containing polyclonal antibodies against cephalexin and cephalotin were tested for cephalexin determination. Tracers based on both CEX and CET and labeled with different fluorescent labels via different functionality were synthesized to achieve the highest possible

Fig. 3 Comparison of the effects of different precipitating agents on the results of measurements

Fig. 4 Linear regression equation derived by use of FPIA data for screening of artificially spiked (**a**) and naturally contaminated (**b**) milk for cephalexin (*n*=5)

A

$y = 1.0141x - 0.2421$
$R^2 = 0.9842$

CEX amount as found by FPIA, µg kg^{-1}

CEX amount, added to sterilized milk sample, µg kg^{-1},

B

$y = 0.7439x + 1.9897$
$R^2 = 0.9834$

CEX concentrartion as measured by FPIA, µu/kg

CEX concentration as measured by HPLC-Fl, µg/kg

sensitivity. The specificity of the FPIA was estimated by calculating the cross-reactivity of the used polyclonal antibodies with a set of cephalosporins and other antibiotics widely used in farming. It was found that the technique is suitable for group analysis of cephalosporins. An extensive investigation was devoted to choice of the optimum sample pretreatment for maximum elimination of matrix interference. Methanol was chosen as optimum protein precipitation agent. The limit of detection of the FPIA for milk was 1 µg kg^{-1}. The technique was used to determine cephalexin in artificially spiked and naturally contaminated milk samples. High-performance liquid chromatography was used to confirm the results.

Conflict of interest The authors declare that they have no potential conflict of interest.

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