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Competitive ELISA of Chloramphenicol: Influence of Immunoreagent Structure and Application of the Method for the Inspection of Food of Animal Origin

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An indirect competitive ELISA for the detection of chloramphenicol (CAP) in food of animal origin (milk, meat, eggs) is described. Influence of immunoreagent structure and composition on the assay sensitivity and specificity was investigated. Two CAP derivatives were used for conjugation with proteins: CAP succinate and a diazo derivative of CAP. Molar incorporation of CAP into the coating conjugates was also varied. To eliminate matrix effect on the assay results, a special casein-containing buffer was used for milk samples, whereas for meat and egg samples a 50-fold dilution of the buffer extracts was needed. The method developed permits CAP concentrations to be determined in the range 0.08–100 μg l^{-1}. The detection limit is 0.08 μg kg^{-1}. Recovery in different food samples averages between 70 and 130%. The method can be applied for inspection of food of animal origin for CAP residues.

Keywords: *Enzyme-linked immunosorbent assay, chloramphenicol, food inspection*

INTRODUCTION

Chloramphenicol (CAP) is a very effective and, therefore, widely used antibiotic for the treatment of a number of infections in cattle, poultry and swine and this has led to concern being voiced over the possibility of CAP residues finding their way into the human food chain. This potential hazard has led to a prohibition of its use in the European Union and to threshold values being set for CAP in edible tissues in many other countries. The maximum residue level (MRL) established in Russia for milk, meat and eggs is 10 μg kg^{-1} (Hygienic regularities, 1997).

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A variety of methods have been developed for the detection of CAP in milk and other samples. A number of chromatographic methods including high-performance liquid chromatography (HPLC) were developed for the quantification of CAP residues (Allen, 1985; Aerts *et al.*, 1989; Long *et al.*, 1990; Moretti *et al.*, 1992). If many samples have to be analysed a reduction in cost and time can generally be obtained by application of a simple and quick screening method such as immunoassay. Radioimmunoassays have been originally applied (Arnold *et al.*, 1984; Arnold & Somogyi, 1985; Hock & Liemann, 1985). Apart from these methods for screening purposes, ELISA (Campbell *et al.*, 1984; Märtlbauer & Terplan, 1987; van de Water & Haugsma, 1987), test kit-based methods (Nouws *et al.*, 1988; Laurensen & Nouws, 1990) or immunofluorescence capillary electrophoresis assay system (Blais *et al.*, 1994) were proposed.

The main difficulty during ELISA performance for food samples is connected with matrix interferences and a low detection limit. In a number of cases, detection down to this level needs clean-up and concentration procedures. A sensitive streptavidin-biotin ELISA for the direct screening of CAP in crude aqueous swine muscle tissue extracts (detection limit 10 $\mu g\,kg^{-1}$; van de Water & Haagsma, 1990a) and in milk (detection limit 1 $\mu g\,kg^{-1}$; van de Water & Haagsma, 1990b) was described. In these papers, the authors proposed to correct for the variable matrix interferences on dose–response curves by treating a part of the sample with an immobilized monoclonal antibody (mAb) preparation, thus removing CAP from the sample, and in this way the response of each sample was compared with the response of its corresponding 'blank'.

The purpose of our work was to study the influence of immunoreagent structure and composition on the sensitivity and specificity of the ELISA of CAP and then to apply this information for developing a method for quantitative detection of CAP in food samples without complicated sample clean-up. The method was based on the principle of indirect competitive ELISA. The advantage of ELISA of this type is that there is no need to obtain enzyme conjugates for every target analyte. Apart from that, enzyme conjugate is used when there is no actual sample in the assay system, so the sample components do not influence the enzyme activity.

MATERIALS AND METHODS

Materials

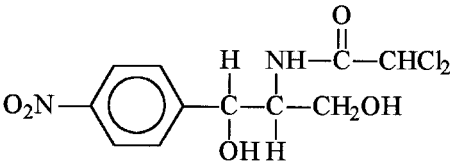
Hydrogen peroxide, *o*-phenylenediamine, trinitrobenzenesulfonic acid and Tween 20 were supplied by Sigma Chemical Co. (St Louis, MO, USA). Methanol, sulphuric acid and inorganic salts were purchased from 'Reakhim' (Moscow, Russia). CAP, CAP succinate and other antibiotics were kindly provided by the National Research Centre of Antibiotics (Moscow, Russia). The water used for dilution and preparation of buffers was obtained by distillation.

The following buffers were used: 0.01 M-KH_2PO_4–$KHPO_4$ buffer, pH 7.4 (PB); PB supplied with 0.15 M NaCl, pH 7.4 (PBS); PBS containing 0.1% Tween 20 v/v, pH 7.4 (PBST); 0.1 M-sodium citrate buffer, pH 5.0 (CB).

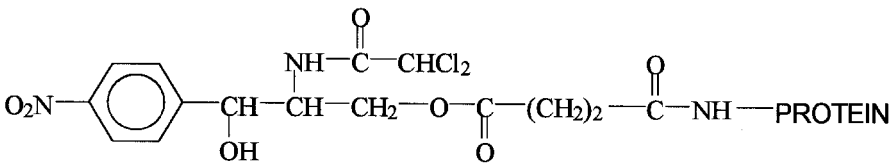
CAP standard solutions were prepared by dilution of the stock solution of CAP in methanol (1 $mg\,ml^{-1}$).

The conjugate of sheep anti-rabbit immunoglobulin with horseradish peroxidase (HRP) was supplied by M. Ph. Gamaleya Institute of Epidemiology and Microbiology (Moscow, Russia).

Polyclonal antibodies (pAb) against immunogen 1 were produced in rabbits (Kolosova *et al.*, 1998) by 'Immunotek' (M. V. Lomonosov Moscow State University, Moscow, Russia). pAbs against immunogen 2 and conjugate ovalbumin (OVA)–CAP 5 (Figure 1, Table 1) were kindly provided by O. V. Aak from the Institute of Antibiotics and Enzymes (St. Petersburg, Russia).

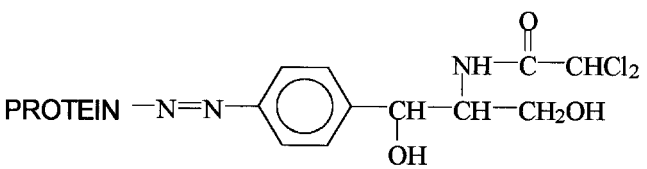


Chloramphenicol (d-threo-isomer)



Immunogen 1

Conjugates OVA–CAP 1–4



Immunogen 2

Conjugate OVA–CAP 5

FIG. 1. Chemical structure of immunoreagents: CAP and CAP–protein conjugates.

Non-contaminated (CAP-free) raw milk was kindly provided by K. A. Timiriazev State Agricultural Academy (Moscow, Russia).

96-well polystyrene strip microtitre plates (high binding) were supplied by Biohit (Helsinki, Finland).

TABLE 1. The composition of coating conjugates

Conjugate[a]	CAP derivative used for conjugation	Moles CAP per mole OVA	
		Reaction mixture	Conjugate
CAP–OVA 1	CAP succinate	100	16
CAP–OVA 2	CAP succinate	50	13
CAP–OVA 3	CAP succinate	25	10
CAP–OVA 4	CAP succinate	10	5
CAP–OVA 5	Diazo derivative of CAP	30	12

[a] The structure of conjugates is given in Figure 1.

Apparatus

Measurements of optical density for 96-well microtitre plates were performed on a microtitre plate reader (Molecular Devices, Palo Alto, CA, USA).

Synthesis of Protein–CAP Conjugates

Protein–CAP conjugates were synthesized by the activated ester method. 700 mg (1.6 mmol) CAP succinate were dissolved in 50 ml distilled water. Then 635 mg (1.5 mmol) 1-cyclohexyl-3-(2-morpholino-ethyl) carbodiimide metho-*p*-toluene sulphonate in 10 ml distilled water and 175 mg (1.5 mmol) *N*-hydroxysuccinimide in 10 ml distilled water were added (pH 5.3). The reaction mixture was incubated with stirring for 1 h at room temperature.

To 200 mg (3 μmol) bovine serum albumin (BSA) in 20 ml PB 20 ml activated CAP succinate (630 μmol) were added. The reaction mixture was incubated with stirring for 2 h at room temperature followed by overnight incubation at 4°C. Subsequently, dialysis against distilled water was carried out for 5 days.

Four solutions of 140 mg (3.1 μmol) OVA in 10 ml PB were prepared. To each solution different amounts of activated CAP succinate were added: 10 ml (315 μmol); 5 ml (160 μmol); 2.5 ml (80 μmol); 1 ml (31 μmol). Reaction mixtures obtained (pH 7.2–7.4) were incubated with constant stirring for 2 h at room temperature, then overnight at 4°C followed by dialysis against distilled water for 5 days. All conjugates were lyophilized.

Molar incorporation of CAP into the protein conjugates was estimated by the titration of free amino groups with trinitrobenzenesulphonic acid (Habeeb, 1966).

Sample Preparation

The samples of milk, meat and eggs were supplied by the Institute of Nutrition (Moscow, Russia).

Milk samples were contaminated by analytical grade purity CAP diluted in ethanol. Milk samples were spiked with different aliquots of antibiotic solution, mixed thoroughly and incubated for 1 h at room temperature.

Meat and egg samples were prepared as follows: 10 g meat (or egg) were homogenized and mixed with 10 ml sterile PBS. The mixture was incubated for 90 min at 37°C with shaking at intervals. The mixture was centrifuged for 20 min at 3000 × *g* and the upper phase was collected.

Food analysis by reverse-phase HPLC was carried out in the Institute of Nutrition (Moscow, Russia) as described in Kirnichnaya & Melamed (1989). Column Ultrasphere ODS 5 μm (250×4.6 mm) and mixture of acetonitryl/water/decylamine (40/60/0.1) as moving phase were used. UV absorption was detected at 278 nm.

Competition ELISA Procedure

The wells of the plates were coated with 100 μl of CAP–OVA 4 at a concentration of 150 ng ml^{-1} in PBS for 2 h at 37°C. The wells were washed with PB (4×300 μl). After drying at room temperature the plates can be stored at 4°C for 1 year. Aliquots of 50 μl CAP standard solutions and sample solutions in PBST were added to coated wells, respectively. Then 50 μl PAb solution at dilution 1/4000 in PBST were added to the wells and the plates were incubated for 1.5 h at 37°C. The wells were washed with PBST (4×300 μl). 100 μl of second antibody–HRP conjugate solution diluted 1/10 000 in PBST were added to the wells followed by another 1-h incubation at 37°C and washing with PBST (5×300 μl). 100 μl fresh prepared substrate solution were added to each well. The substrate solution contained 4 mg *o*-phenylenediamine and 4 μl hydrogen peroxide (30% (v/v)) per 10 ml CB. The colour reaction was stopped after 25–30 min (for milk samples) or 7–10 min (for meat and egg

samples) using 50 µl 4M H_2SO_4 solution. The optical densities were read at 492 nm. The values of optical densities were converted to $\%B/B_0$ values according to the formula:

$$\%B/B_0 = \frac{A-A_{excess}}{A_0-A_{excess}} \times 100,$$

where A is the value of optical density for the sample or standard, A_0 is the value of optical density for 'zero' standard, A_{excess} is the value of optical density for the sample, containing excess of analyte (CAP).

Cross-reactivities were determined by comparing middle points of the assay for different compounds:

$$\%\text{ cross-reactivity} = \frac{\text{analyte concentration at } 50\%B/B_0}{\text{concentration of cross-reacting compound at } 50\%\ B/B_0} \times 100.$$

Average dissociation constants for the complex of CAP with pAbs were determined by the ELISA procedure (Friquet *et al.*, 1985) using Klots coordinates for the experimental data linearization.

RESULTS AND DISCUSSION

Influence of Immunoreagent Structure and Composition on the Basic Characteristics of Immunoassay

Two CAP derivatives were used for conjugation with proteins: CAP succinate and the diazo derivative of CAP (Figure 1). Immunogen 1 and conjugates CAP–OVA 1–4 were synthesized from CAP succinate, and immunogen 2 and conjugate CAP–OVA 5 obtained by conjugation of the protein molecule via the nitro group of the antibiotic, from the diazo derivative. Molar incorporation of CAP into the coating conjugates CAP–OVA 1–4 obtained from CAP succinate was varied from 16 to 5, respectively (Table 1).

Different polyclonal antisera obtained against the immunogens 1 and 2 were tested by the ELISA using homologous coating conjugates with a maximum molar incorporation of CAP into OVA (conjugates 1 and 5, respectively, Figure 1, Table 1). For further investigations, antisera with the best titres were used.

It was demonstrated that for the antiserum against immunogen 2 synthesized from the diazo derivative of CAP, specific binding was observed both with conjugate CAP–OVA produced from the diazo derivative of the antibiotic (homologous pair of reagents) and with the conjugates CAP–OVA synthesized from CAP succinate (heterologous pair of reagents), whereas for the antiserum against immunogen 1 obtained from CAP succinate – only with conjugates CAP–OVA homologous to the immunogen did binding occur (data not shown). It has been known from literature that the dichloroacetamido and nitrophenyl groups of CAP molecule account for a large portion of the immunological reactivity of the hapten (Hamburger & Douglass, 1969). Antibodies obtained via the immunogen 1 (Figure 1) synthesized from CAP succinate are probably produced not only against the dichloroacetamido group of CAP molecule, but to a large extent, against the nitrophenyl group. Thus, such an antiserum had no specific binding with the heterologous conjugate CAP–OVA 5, in which the nitro group of CAP molecule was used for conjugation with the protein. Evidently, the presence of the nitro group in the CAP molecule has a dramatic effect on the interaction with antibodies.

Furthermore, with different concentrations of free CAP added in the assay system, the competitive stage of the ELISA was studied (Figure 2). In the case of the antiserum against immunogen 2, the structure and composition of the conjugates CAP–OVA didn't influence

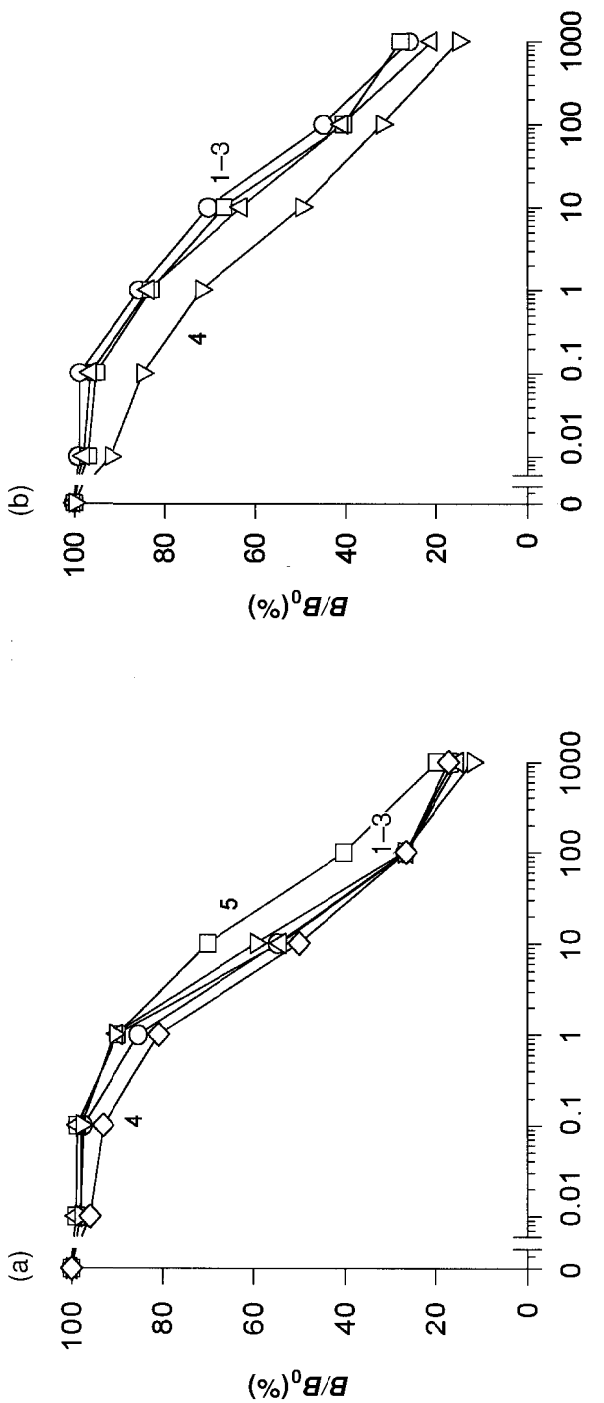


FIG. 2. Dose–response curves of CAP standard solutions for different combinations of antisera and coating conjugates. (a) Antiserum against immunogen 2; (b) antiserum against immunogen 1. Curve numbering corresponds to conjugate numbering (Table 1).

markedly the position of the dose–response curve (Figure 2(a)). The antiserum against immunogen 1 being used (Figure 2(b)), for homologous to the immunogen conjugates CAP–OVA 1–3 with 16–10 molar incorporation of CAP (Table 1), there were no considerable variations in the position of dose–response curves (curves 1–3). When passing to conjugate CAP–OVA 4 with a minimum molar incorporation of CAP, the dose–response curve shifted to the lower CAP concentrations region (curve 4). Obviously in this case the CAP–OVA conjugate is most readily displaced from the antigen–antibody complex by free CAP resulting in an increase in assay sensitivity.

For the antiserum against immunogen 2 assay sensitivity was demonstrated to be higher for the heterologous assay than for the homologous (Figure 2(a), curves 4 and 5, IC_{50} 10 and 50 ng ml^{-1}, respectively). For dose–response curves obtained with the use of homologous pairs of reagents it is worth noting that for the pair, antiserum against immunogen 1–conjugate CAP–OVA 4, the dose–response curve shifted to the range of lower CAP concentrations (Figure 2(b), curve 4, IC_{50} 10 ng ml^{-1}) in comparison with the pair, antiserum against immunogen 2 – conjugate CAP–OVA 5 (Figure 2(a), curve 5, IC_{50} 50 ng ml^{-1}). In this case the antisera quality and affinity is likely to be of critical importance. For both antisera average dissociation constants for the complex of CAP with polyclonal antibodies were determined by the ELISA procedure (Friquet *et al.*, 1985). The constants were equal to 5.6×10^{-9} M for the antiserum against immunogen 1 and 5.4×10^{-8} M for the antiserum against immunogen 2.

Data on specificity of antisera against immunogens 1 and 2 with different structure were obtained. For the compounds structurally related to CAP, cross-reactivity was observed only for CAP succinate (1428 and 24%, respectively). Extremely high specificity of the antibodies against immunogen 1 with respect to this compound (more than 10 times higher than to CAP) results from the fact that this immunogen was synthesized by conjugation of BSA with CAP succinate. It is worth mentioning that for the antibodies against immunogens 1 and 2, cross-reactivity for deacylated CAP, which is one of the metabolites of CAP, was less than 1%. For other antibiotics (aminoglycosides, β-lactam antibiotics, tetracyclines, macrolide antibiotics) cross-reactivity was less than 0.1%.

Hence immunoreagent structure and composition have significant influence on the assay characteristics, and this information can be used for the development of an ELISA for CAP detection depending on the particular research and practical purposes. So for CAP quantification in human blood serum (Kolosova *et al.*, 1998) when the antibiotic is to be detected in the therapeutic range (5–30 μg ml^{-1}) which is higher than the determinable range of CAP concentrations, the antiserum against immunogen 2 and the conjugate CAP–OVA 5 were used, since the linear range of dose–response curve corresponding to this homologous pair of reagents shifted to the region of higher CAP concentrations (Figure 2 (a) curve 5).

ELISA for Chloramphenicol Residues in Food of Animal Origin (Milk, Meat, Eggs)

The main problem during optimization of the method for CAP detection in food was to attain the low detection limit determined by the MRL of CAP in food of animal origin (10 μg kg^{-1}), and to correct for variable matrix interferences. Homologous assay with the use of antiserum against immunogen 1 and conjugate CAP–OVA 4 was applied for this purpose, as this pair of reagents was shown to provide the best assay sensitivity.

Much attention was given to the investigation of matrix effect. Influence of matrix interferences in milk on the assay measurements was studied in detail. Successive dilution of milk with the buffer did not result in successive shifting of dose–response curves. Milk being diluted 1/100, the dose–response curve turned out to be below the corresponding curves for CAP solutions in milk, diluted 1/1000 and 1/10 (data not shown). This fact is likely to be ascribed to the complicated matrix of milk samples. At the same time the values of optical density for CAP standards in milk diluted 1/100 were two times lower than for CAP standards in buffer. Stirring during the competitive stage of the ELISA did not eliminate this

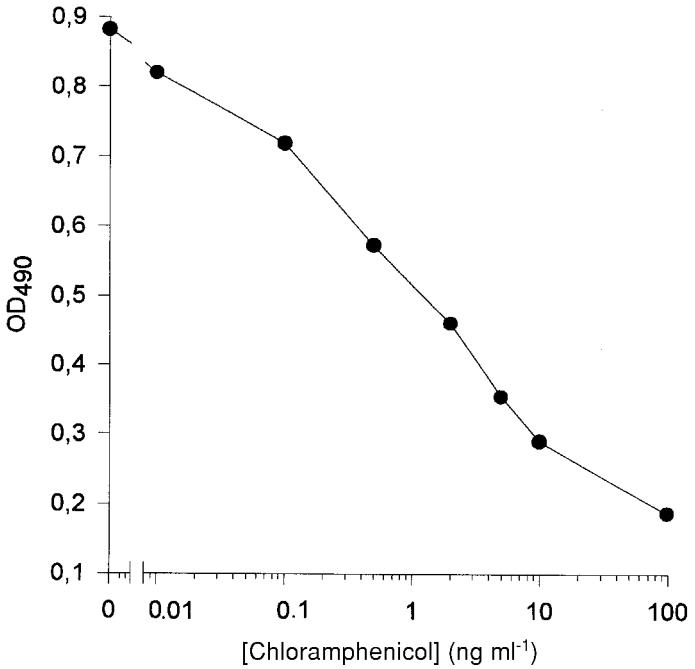


FIG. 3. Dose–response curve of CAP standard solutions in milk.

effect. Thus even 1000-fold milk dilution did not eliminate matrix effects. In this connection an effort was made to develop an analytical system which would allow to eliminate matrix components influence on the assay measurements. To solve this problem two main approaches could be used: (1) clean-up of the sample or (2) using reagents and buffers which would permit to correct for matrix interferences. But clean-up is rather time-consuming and it would make the analysis more complicated, so the second approach was used.

It is common knowledge that the main milk protein is casein (80% of total protein amount), which content is of 2.8–3.5% by mass, so casein was added to the assay buffer. Influence of casein concentration in the buffer on the analytical system was described in our previous work (Kolosova *et al.*, 1999). Casein concentration in the assay buffer constituted 1%, which far exceeded casein content in milk samples which are to be diluted during the assay procedure. It was shown then that using this buffer for the preparation of CAP standard solutions and 100-fold dilution of milk with the same buffer allowed to obtain the uniform dose–response curve independently of milk composition and treatment and therefore led to elimination of matrix interferences. Using antibodies with high affinity (antiserum against immunogen 1 and coating conjugate CAP–OVA 4 (Figure 1, Table 1) enabled to attain a low detection limit (0.08 $\mu g\ l^{-1}$; Figure 2(b)), and milk samples could be diluted 1/100 during the assay. As a result of this, a calibration curve for the detection of CAP residues in milk was obtained (Figure 3). The method developed permits CAP concentrations to be determined in the range of 0.08–100 $\mu g\ l^{-1}$ in different kinds of milk including dry milk products. The detection limit is 0.08 $\mu g\ l^{-1}$ in milk diluted 1/100. The assay is characterized by good reproducibility (CV ranged from 6.0 to 10.8% for inter-assay, and from 2.2 to 10.4% for intra-assay, Table 2).

To validate the method reliability, correlation between ELISA and HPLC for CAP detection in milk was carried out. Forty-six spiked samples of different kinds of milk including sterilized milk with different fat content, raw (untreated) milk and dry milk

TABLE 2. Analytical characteristics of the ELISA method for CAP determination in food samples

Parameter		Value
Detection limit ($\mu g\ l^{-1}$)		0.08
Coefficients of variation (%)	Intra-assay	6.0–10.8
	Intra-assay	2.2–10.4
Recovery (%)	Milk	70–130
	Meat, eggs	85–130

products were tested. Milk was spiked at 1, 5, 10, 20, 100, 200, 1000 and 10 000 $\mu g\ l^{-1}$ levels. After 100-fold dilution each sample was submitted five-fold to the ELISA. Good correlation was observed ($y = 0.01 + 0.68x$, $r = 0.977$). False-negative and false-positive results were not revealed during the analysis. Recovery in milk for ELISA averaged between 70 and 130%.

The method was applied also for the detection of CAP residues in eggs and meat. Aqueous extracts of these products were used for the ELISA. In this case the matrix effect was characterized by ordinary regularity: successive dilution of meat extract with the assay buffer resulted in successive shifting of dose–response curves (results not shown). 100-fold dilution of the samples (extracts of poultry meat) allowed to eliminate matrix components influence on the assay results. A similar picture was observed for egg extracts. Extracts of different samples of poultry meat from local butchers and eggs were spiked at different CAP levels and after 50-fold dilution (total dilution 1/100) were submitted five-fold to the ELISA. Recovery in poultry meat and eggs extracts analysed by ELISA averaged between 85 and 130% (Table 2), what is considered to be acceptable for food inspection, because in this case there is no need to obtain extremely high precision for CAP quantification, and the samples being analysed differed greatly in the composition and the way they were treated. The detection limit was 0.08 $\mu g\ l^{-1}$ in the extract with total dilution 1/100 or 8 $\mu g\ kg^{-1}$ in meat or eggs. When the samples of meat of other animals (swine, cattle etc.) were analysed, even 100-fold dilution of buffer extracts did not result in the elimination of matrix interferences. In this case the probability of false-positive results occurring is high, so more complicated clean-up may be required. This problem is under investigation now.

To demonstrate the applicability of ELISA for the detection of CAP residues in real samples, a rabbit (4 kg) was given intramuscular injections of CAP succinate on 3 successive days: day 1, one injection of 500 mg; day 2 two injections of 1000 mg each; day 3, two

TABLE 3. ELISA and HPLC results of the determination of CAP residues in the tissues of control and CAP-treated rabbits

Sample		CAP concentration ($\mu g\ kg^{-1}$)	
		ELISA	HPLC
Muscle (front leg)	Control	< 10	< 10
	CAP-treated	710 ± 50	300 ± 40
Muscle (hind leg, place of injection)	Control	< 10	< 10
	CAP-treated	> 10 000	> 10 000
Liver	Control	< 10	< 10
	CAP-treated	27.7 ± 2.7	76
Kidney	Control	< 10	< 10
	CAP-treated	> 10 000	> 10 000

injections of 1000 mg each. Altogether, within 3 days 4500 mg of CAP or 1125 mg per kg body weight was administered. The test animal and an uninjected control rabbit were slaughtered 18 h after the last administration. The samples of muscle tissues of front leg and hind leg (place of injection), liver and kidney tissues were submitted to the ELISA as described. Assay results were compared with those obtained by HPLC (Table 3). Both methods gave adequate results, but trace amount of the antibiotic was revealed in the control samples by the ELISA, so more thorough clean-up of the samples of animal tissues is thought to be appropriate.

CONCLUSION

Influence of immunoreagent structure and composition on the sensitivity and specificity of ELISA for CAP was studied. This information can be used for the development of immunoassay for CAP detection in different matrices depending on the particular research and practical purposes. The method of indirect competitive ELISA for the determination of CAP in food samples (milk, meat, eggs) was developed. CAP concentrations in milk or food extract determinable by the ELISA range from 0.08 to 100 $\mu g\,kg^{-1}$. Detection limit is 0.08 $\mu g\,kg^{-1}$. Coefficient of variation (intra- and inter-assay) is less than 12%. Milk and food extracts (meat and eggs) are diluted 1/100 in the analysis. The method can be applied for inspection of food of animal origin for CAP residues.

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