LABORATORNÍ PŘÍSTROJE A POSTUPY

THE USE OF ELISA METHOD FOR THE DETERMINATION OF CHLORAMPHENICOL IN FOOD PRODUCTS OF ANIMAL ORIGIN

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Contents

1. Introduction

The antibiotic chloramphenicol (CAP) is often used in medicine and veterinary medicine. Chloramphenicol is recognised as a very effective antibiotic as it can liquidate many types of microflora; however, it has also toxic effects on the human organism. The toxic action of chloramphenicol is specifically similar to that of carcinogenic compounds[1]. Although these properties have not been completely proved so far, they should not be underestimated since they are based on reliable studies[2]. In order to determine its real occurrence in terms of form and amount, numerous analytical and research studies have been undertaken[3-6].

The analytical problem in the determination of chloramphenicol in trace quantities results from the detection limit of the variety of forms appearing in food[7,8]. The well-known spectrophotometric[9], fluorometric[10], PC and TLC[11] methods fail to record any signals of chemical reaction. Most of the methods mentioned are not or need not be based on a chemical reaction with this compound present in low quantities. So far the analysis of CAP in meat and its products has been based mainly on HPLC (Ref.[12-14]), GC (Ref.[15]) and mass spectrometry[16,17]. Special HPLC, GC and MS instruments suitable for such analyses are available only in selected specialist research centres. In addition, these measurement techniques require expensive special equipment. In these methods, another unquestionable problem consists in the procedure of sample preparation for analysis. Mostly, extraction processes with organic solvents are used[18]. In the last 10 years, chromatographic techniques in combination with enzymatic and immunochemical methods have been developed, which may have practical use in the determination of CAP in food[19,20].

The aim of this study was to determine the chloramphenicol content in selected food products of animal origin and to evaluate suitability of the ELISA method for the determination of chloramphenicol in food of everyday consumption.

2. Experimental

2.1. Materials

The following equipment was used in the studies: HP 8453 UV-VIS spectrophotometer (Hewlett Packard), ELISA reader (MAGPOL – Wrocław, Poland), microcentrifuge, type 310, ("Mechanika Precyzyjna" – Warsaw, Poland), CAT X-120 high-speed homogeniser (Cole-Parmer, USA), WPE 30S balance (RADWAG, Poland) for the determination of dry weights of samples at 50–140 °C, KBC-G-100/250 incubator (PREMED, Poland), 8-channel washer (BIOTEK Instruments Inc., USA), 1-channel micropipettes 10–500 µl (Plastomed, Poland) and 8-channel micropipettes 5–50 µl and 50–250 µl (Sigma, USA).

The immunoenzymatic analysis was carried out on a polystyrene microplate with 96 microwells of flat bottoms.

Standards of chloramphenicol of Riedel-de Haën (Germany) were used as solutions of the following concentrations: 0, 10, 25, 50, 100, 200, 500 ng.dm^{-3}. There were also used:

– specific antibodies against chloramphenicol,
– chloroamphenicol-peroxidase conjugate,
– 3,3',5,5'-tetramethylbenzidine as a substrate (TMB),
– phosphate buffer of pH 7.0, using an addition of Tween 20 as a solution for microwell washing, and
– 1 mol.l^{-1} sulfuric acid as a quenching reagent. All the reagents were of Riedel-de Haën (Germany).

In addition, there were used: chloramphenicol standard, Na_2HPO_4, KH_2PO_4 and NaCl, all Analar grade of Merck (Germany). The following solvents were used: ethyl acetate, methanol, ethanol, acetone, acetonitrile and chloroform (POCH – Poland). The enzymes used included: leucine aminopeptidase (EC 3.4.11.1) and acylase I (EC 3.5.1.14) of Sigma (USA). The water used in experiments had a conductivity below 0.01 µS.

2.2. Procedure

Determinations were carried out with the use of the immunoenzymatic method ELISA based on the formation of specific antigen-antibody complex[4]. The microwells covered with antibodies against chloramphenicol in 0.01 mol.l^{-1} phosphate buffer with 0.15 mol.l^{-1} NaCl (pH = 7.3) were incubated at room temperature (20 °C) and then filled with 100 µl of

chloramphenicol standard solutions or samples to be analyzed for the chloramphenicol content. Then, 50 µl of the conjugate solution marked with peroxidase was added. After 4 h incubation at room temperature, the microwells were emptied and washed 5–6 times with phosphate buffer solution containing Tween 20. Then the moisture residues were removed by gentle tapping the plates against a soft paper. Immediately after washing, the microwells were filled with 100 µl of substrate 1 mmol.l^{-1} solution of substrate and incubated for 30 min in the dark at room temperature. The enzymatic reaction of peroxidase, H_2O_2 and 3,3',5,5'-tetramethylbenzidine (TMB) resulted in a blue product. Adding 100 µl of the quenching reagent solution terminated the enzymatic process. The product changed its colour into yellow. After thorough mixing by swinging and gentle shaking, the absorbance of solution was measured at a wavelength of 450 nm. The value of absorbance was inversely proportional to the concentration of chloramphenicol in the sample tested. The procedure of determination by the immunoenzymatic method is schematically shown in Fig. 1.

2.3. Sample preparation

Samples of meat were disintegrated and homogenised, while those of milk, eggs and their products were homogenised. The disintegrated samples were used to prepare portions of about 2 g weight with an accuracy of 1 mg, then phosphate buffer pH 7.0 was added and the mixture was shaken in a thermoshaker for 30 min at room temperature.

All the operations of sample preparation may be divided into three characteristic stages, schematically shown in Fig. 2. The extraction of the analysed substance for all the examined samples was carried out using five variants. Two of them consisted in enzymatic hydrolysis of homogenates. In the first, the samples were hydrolysed with the use of leucine aminopeptidase (LAP) and in the other, the samples were hydrolysed with acylase I (AcI). In remaining three variants, the samples were directly extracted with methanol, ethanol or ethyl acetate. The samples tested in the first and second variants were, after 3 h hydrolysis, incubated at 100 °C for 15 min to terminate this process. After cooling to room temperature, ethyl acetate was added to the analysed extract. Then all the samples were shaken in the thermoshaker for 20 min and centrifuged for 20 min at 3000 rpm. After decantation of the supernatant, another portion of the solvent was added to the residues in test tubes and shaking was repeated for 15 min followed by centrifuging at 5000 rpm for 15 min. The combined supernatants were evaporated on a water bath to remove the solvent. When the sample volumes were reduced to 1 ml, another 1 ml of the solvent was added followed by evaporation to dryness. After cooling to room temperature, the solid residue was dissolved in 1.0 ml of ethanol and analyzed.

3. Results and Discussion

The test for the chloramphenicol content involved basic food products of animal origin such as cured pork meat, turkey breast, chicken breast, sirloin and rump, pork shoulder, pork liver and kidney, full fat milk, granulated dried skim milk, eggs and mayonnaise.

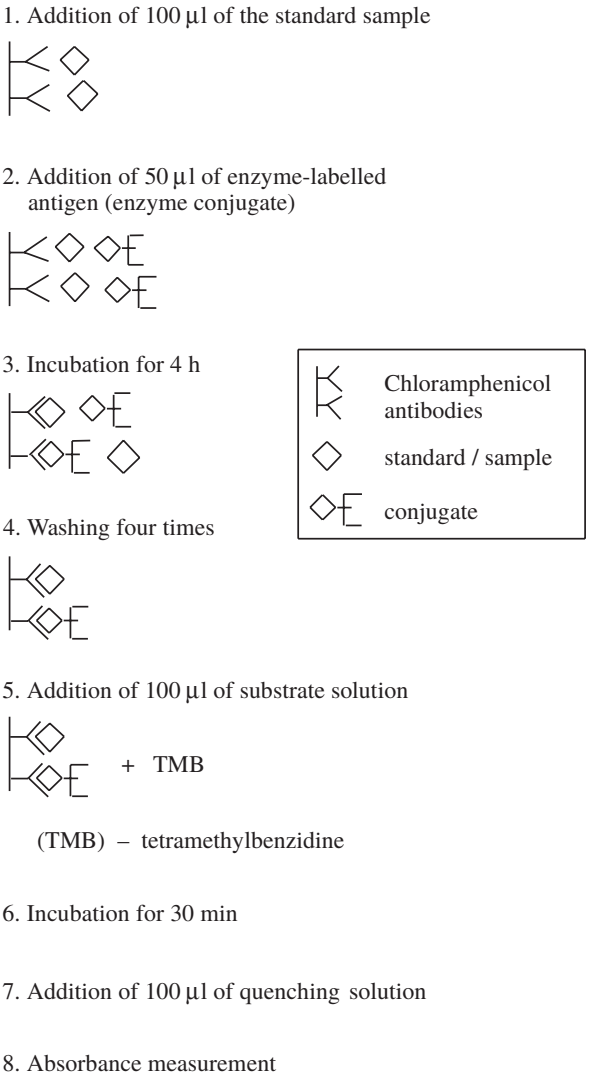

Fig. 1. **Analysis scheme of the determination of chloramphenicol in food samples by the ELISA immunoenzymatic method**

Chloramphenicol and its derivatives may appear in food products in various forms. It was intended to perform the analysis of the same samples using various procedures.

A significant point of the total analysis was to determine the dry matter in the samples tested. The determination was carried out in two ways. The first consisted in the conventional multiple drying of samples at 100 °C to a constant weight. In the other, a special balance was used to, performing the operation at two different temperatures, 110 and 130 °C for each sample. The calculated water contents in samples are given in Table I.

Figure 1 shows the ELISA procedure used for the determination of chloramphenicol in food samples which were prepared using five different variants. Two of them comprised hydrolysis and release of chloramphenicol from more complex systems. The hydrolysis of acyl or peptide bonds was carried out with the enzymes used previously[21]. Owing to the cleavage of such bonds, the amine groups of chloramphenicol are

exposed and they can take active part in the combination with antibodies against chloramphenicol used in ELISA. After the enzymatic hydrolysis, the compounds of chloramphenicol were extracted with ethyl acetate, which was evaporated after repeating the process. The solid residue dissolved in ethanol was used for determinations. The remaining three variants of sample preparation comprised direct extractions with various organic solvents, methanol, ethanol, or ethyl acetate. The whole procedure of sample preparation is illustrated in Fig. 2. Generally, the sample preparation procedure is performed in three stages, including: 1 – disintegration and homogenisation, 2 – double extraction with enzymatic treatment if necessary,

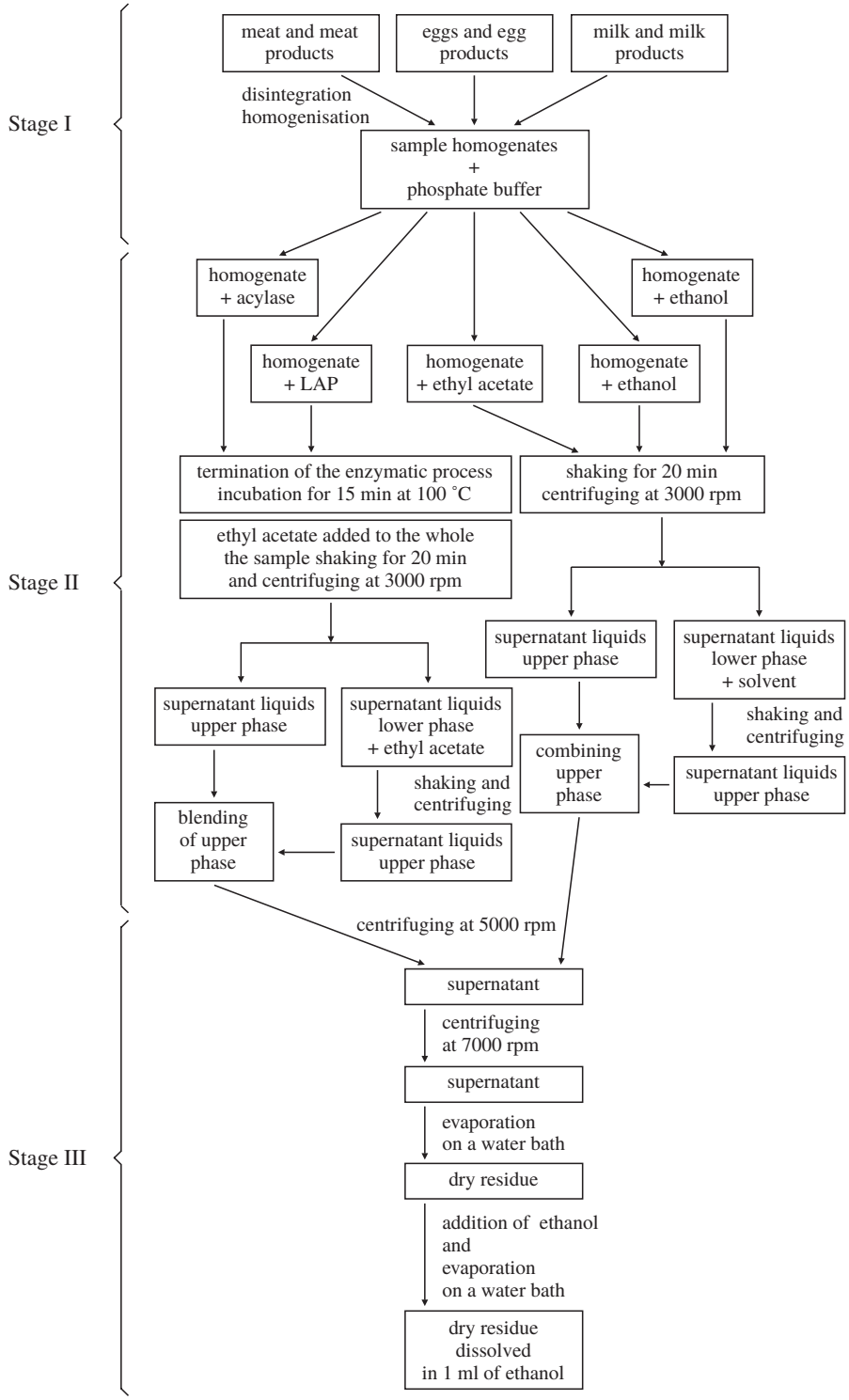

Fig. 2. **Scheme of the procedure of food sample preparation for the determination of chloramphenicol by the immunoenzymatic method**

Table I
Chloramphenicol content in food product determined by ELISA method

No	Sample	Dry matter [%]	Chloramphenicol ng/g dry matter					CAP content (n = 3) in EtOAc ng/g dry matter	
			EtOH	MeOH	EtOAc	LAP	Acyl	without hydrolysis	with hydrolysis
1	Chicken	24.68	20	88	132	346	352	132(±6)	349(±4)
	breast		18	109	144	288	318	144(±8)	303(±8)
2	Pork shoulder	27,11	158	126	109	229	230	109(±3)	229(±1)
3	Sirloin	24.99	180	184	121	–	387	121(±6)	387(±8)
			–	–	138	392	400	138(±5)	396(±4)
4	Rump	29,88	44	15	–	125	140	–	132(±8)
			–	–	104	127	138	104(±4)	132(±8)
5	Pork kidney	23.04	323	142	180	–	–	180(±5)	–
			–	–	152	372	379	152(±8)	375(±7)
6	Pork liver	31.08	84	80	98	–	–	98(±4)	–
			–	–	94	204	208	94(±4)	206(±3)
7	Turkey breast	24.31	9	8	12	–	–	12(±3)	–
			–	–	13	26	22	13(±2)	24(±3)
8	Ham in bladder	28.94	8	7	9	–	–	9(±4)	–
			–	–	13	54	37	13(±4)	45(±5)
9	Granulated dried skim milk	98,48	107	102	111	124	137	111(±3)	130(±5)
10	Full fat milk	10.00	40	50	100	102	104	100(±5)	103(±6)
11	Eggs	43.12	9	16	19	21	25	19(±3)	23(±4)
12	Mayonnaise	54.41	7	15	18	20	22	18(±3)	21(±3)
13	Full fat milk	10,00	151	138	227	362	368	227(±5)	365(±5)

3 – solvent evaporation and washing followed by the final dissolution of the solid residue.

Figure 3 shows a standard curve obtained for standard solutions of chloramphenicol of concentrations 0, 10, 25, 50, 100, 200 and 500 ng.dm^{-3}. The curve represents a sigmoidal relationship % A = f(c_{CAP}), where % A is the ratio of absorbance of the tested sample to test of the blank. For some samples, the reading of absorbance was impossible due to the fact that the chloramphenicol content was beyond the analysed concentration range. The results of chloramphenicol determinations in various food products are given in Table I. Considering these results, it should be stated that high contents of chloramphenicol are found in samples of milk and meat products such as chicken breast, sirloin, pork shoulder and kidney. In the remaining cases, the chloramphenicol content was intermediate, with the lowest quantities found in eggs and mayonnaise.

Analysing the CAP contents in the tested samples in dependence on the solvent used, one can clearly notice higher contents in the extracts where ethyl acetate was used as a solvent in comparison with methanol or ethanol extracts. With samples subjected to the enzymatic hydrolysis, the results obtained are twince or three times higher than those with the samples extracted with ethyl acetate without preliminary hydrolysis. It is likely that in the hydrolysis, chloramphenicol in the form of esters, amides or imines is released. Chloramphenicol appears in tissues in the form of palmitate or succinate esters[1,5]. In addition, in such systems, other unknown substances may be released and combined in a way similar

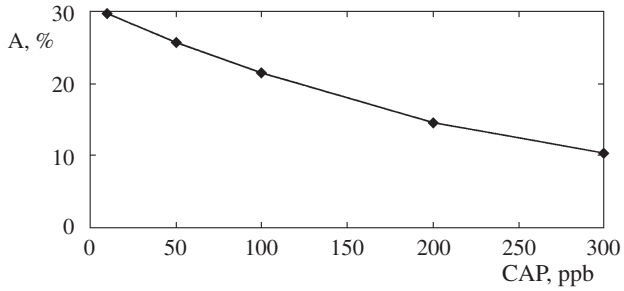

Fig. 3. **Standard curve used for the determination of chloramphenicol (CAP) in meat products by the ELISA methods**; A – absorbance at λ = 450 nm

to that of antibodies used in ELISA. Specific antibodies against the chloramphenicol should not react with other compounds present in the system, but CAP analogues may form in the hydrolysis. Hence, the present procedure of sample preparation using enzymatic treatment requires further investigation.

4. Conclusion

The obtained results indicate the possibility of using the ELISA method for the determination of chloramphenicol in meat and meat products. The proposed method enables the determination of chloramphenicol in the concentration of 0.5 ppb in tested samples. The detectability limit obtained confirms

a high sensitivity of the immunoenzymatic method. The proposed method is fast, inexpensive and reproducible.

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The Use of ELISA Method for the Determination of Chloramphenicol in Food Products of Animal Origin

A sensitive, competitive ELISA method (enzyme-linked immunosorbent assay) for the determination of chloramphenicol (CAP) in food products of animal origin is proposed. The method involves extraction of CAP from samples containing methanol, ethanol or ethyl acetate. In one series of samples, enzymatic hydrolysis of homogenates was used. The procedure, selectivity and sensitivity of immunoenzymatic determinations using an ELISA system are described and discussed. In the tested food products, the presence of CAP from 10^0 to 10^2 ng per g of dry matter was found. The limit of CAP determination in the analysed samples was 0.5 ng.