Teagasc
Agriculture and Food Development Authority

FINAL REPORT

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Methods for Antibiotic Residues in Food

The National Food Centre
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Contents

METHODS FOR ANTIBIOTIC RESIDUES IN FOOD

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INTRODUCTION

Consumer demands for the production of residue-free foods, the desire of regulatory agencies to protect the consumer, and the requirements of the international food trade that foods comply with established limits for residues, have generated a need for increased residue testing of food products. Therefore, there is a demand for simple, rapid and robust methods to test food for residues.

Antimicrobials are widely used in animal production for the treatment and prevention of disease. Antibiotic testing is critically important to the meat and dairy industries, in that both product manufacturing and the acceptability of these products in the market place are dependent on residue status.

The objectives were to develop technologies for the extraction and determination of antibiotic residues in food and particularly to investigate the potential use of alternative immunoassay-based systems. It was also the aim to develop prototype kit systems from the developed methods, to evaluate these at laboratory level and subsequently evaluate their performance for industrial application.

SUMMARY

- Screening methods were developed and validated for the rapid extraction and detection of selected antibiotic residues, including sulphamethazine (SMZ), in meat products at concentrations above the maximum residue limit (MRL) of 100 ppb (parts per billion).
- An extraction procedure for SMZ from pork tissue was developed and validated. The method was based on matrix solid phase dispersion (MSPD) coupled to solid phase extraction (SPE). This method gave recovery of residue from meat samples of greater than 90%. The method was modified to incorporate the use of commercial SPE columns in the procedure. Filtration of pork extracts facilitated the use of immunoassay-based systems for SMZ determination. The preliminary immunoassay system evaluated

was a latex agglutination inhibition assay (LAIA) which requires minimal training and gives a response in 5 minutes. Subsequently, an alternative sol particle immunoassay (SPIA) was evaluated as a detection system and also gave a rapid response time of 15 minutes.

- The complete MSPD/SPE/LAIA method was validated and the total method was developed into a prototype kit, which was evaluated in the laboratory. The kit was then evaluated in two pork processing plants; it was shown that all samples containing no SMZ were correctly classified as "negative" and that all the samples containing SMZ at greater than the MRL were classified as "query positive" or "positive".
- The SPIA detection method was tested as an alternative to the LAIA method and was shown to be easier to use. When coupled with the extraction procedure, the MSPD/SPE/SPIA method was tested successfully both in the laboratory and in a pork processing plant.

ANALYTICAL METHODS FOR SULPHAMETHAZINE TESTING

Sulphonamide antibiotics are a group of drugs widely used in veterinary practice for the treatment of infections in pigs. SMZ is one such drug commonly used as a feed additive. Owing to the potential for misuse, such as an inadequate withdrawal period prior to slaughter, rapid and sensitive procedures are required for the determination of SMZ in meat at levels at or below the maximum residue limit (MRL) of 100 ppb. However, many methods currently used for the extraction, clean-up and detection of SMZ in pork are time consuming and are expensive to perform in large quantities. In many cases, they are only suitable for use within an experienced laboratory. Therefore, there is a need to develop kit systems which are rapid and easy-to-use and would allow for on-site screening of residues in animal tissues at the slaughter plant.

The National Food Centre developed the residue extraction technologies and the National Diagnostics Centre, National University of Ireland, Galway, developed, optimised and produced pilot scale immunoassay-based diagnostic test systems.

Matrix solid phase dispersion and solid phase extraction

MSPD is a suitable extraction procedure for SMZ in pork (Shearan and O'Keeffe, 1994). This method was adapted by coupling of the MSPD step with a SPE clean-up and concentration step, followed by analyte determination, as outlined in Figure 1. During method optimisation the determination procedure used was high performance liquid chromatography (HPLC). New sorbents for the MSPD step were evaluated and a C18 sorbent designed specifically for the MSPD procedure (Horne et al., 1998) functioned better than the general C18 sorbent. This sorbent blended more homogeneously with the pork tissue, resulting in better packing characteristics when formed into a column for washing and elution of analytes.

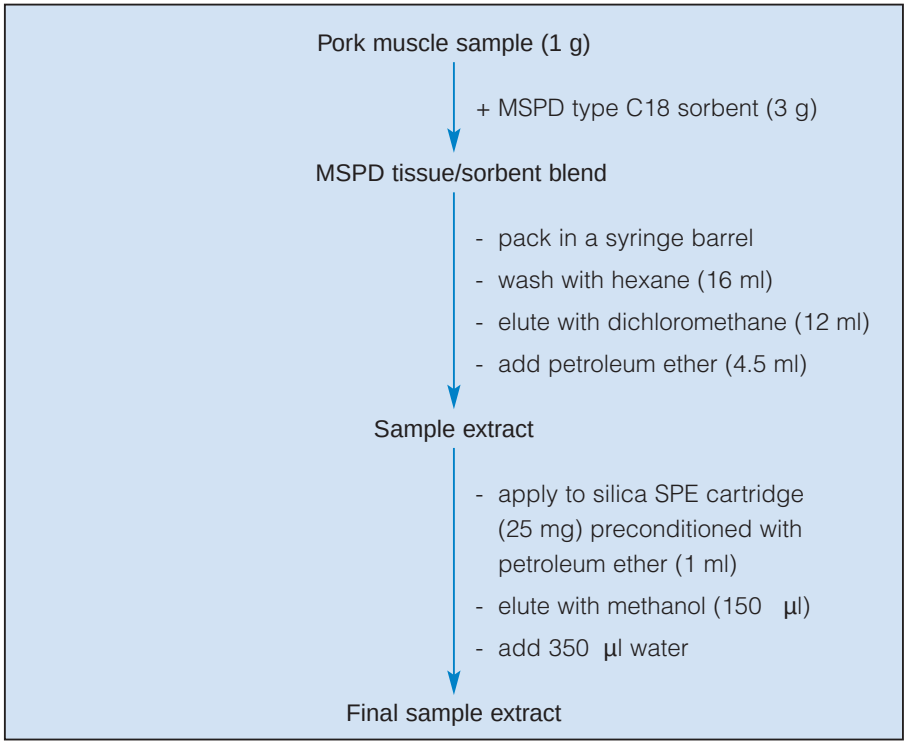

Figure 1: Extraction procedure for sulphamethazine from pork muscle.

Further work on the silica SPE step for concentration of the MSPD tissue extract was carried out. It was observed that laboratory prepared microcolumns gave variable results and the use of commercial SPE microcolumns gave increased consistency. A range of elution conditions from the silica microcolumns were applied and 150 µl of methanol was the optimal volume of elution solvent.

The performance of the MSPD/SPE method was assessed through recovery studies, i.e. addition of a known quantity of SMZ to a residue-free muscle sample and determination of the amount of the added residue recovered by the method. The method was tested at two levels of fortification (25 ppb and 125 ppb). Mean recoveries of between 90% and 120% for both levels of fortification indicate good performance of the method (Figure 2). The repeatability of the method at both levels of fortification is satisfactory with coefficients of variation (CV) of less than 10%. The reproducibility of the method was also shown to be satisfactory (CV below 10%).

The method was further evaluated by the analysis of incurred samples. Incurred samples were obtained by treating pigs with SMZ and taking muscle samples at slaughter. Four incurred samples were generated containing SMZ in the range of 25 to 300 ppb and acceptable results were achieved, indicating the suitability of the method for testing residue-positive pork at concentrations above and below the MRL (Figure 3).

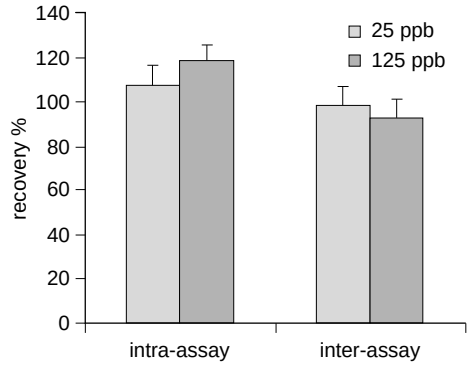

Figure 2: Recovery of sulphamethazine from fortified pork samples by MSPD/SPE.

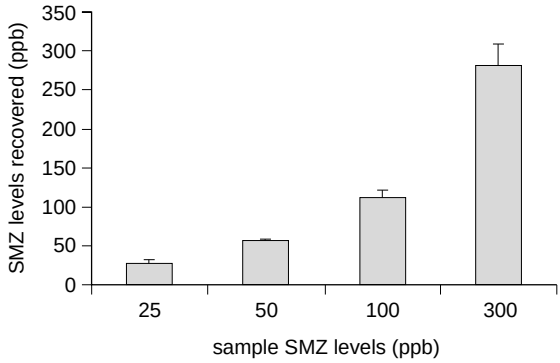

Figure 3: Recovery of sulphamethazine from incurred pork samples by MSPD/SPE.

The method also underwent a rigorous robustness testing, where critical steps in the procedure were examined to establish the effect of variation on the performance of the method. This evaluation is important to establish whether the method is likely to perform well in different laboratories under different conditions, which is an essential characteristic of a kit test. The critical steps evaluated included variation of the amount of sorbent used in the MSPD step, variation in the volume of solvent used for elution from the silica SPE column, and performance of the procedure by different analysts. The results of this robustness testing combined with the method validation indicated that the MSPD/SPE procedure was suitable for use as part of a kit method for SMZ screening in pork samples.

Latex agglutination inhibition assay

A polyclonal antibody to SMZ was sourced at the National Diagnostics Centre and a latex agglutination assay was developed (Foran et al., 1998). SMZ was conjugated to bovine serum albumin (BSA) and this was coated onto polystyrene (latex) microparticles. When the coated particles were mixed with antibody, a visible agglutination was observed. This agglutination is inhibited in the presence of SMZ, such as the addition of a residue positive sample extract. Conversely, in the absence of SMZ, agglutination of the latex particles can be seen, which indicates a negative sample. The LAIA technique is outlined in Figure 4.

The performance of the LAIA for SMZ was examined over a range of conditions, with the goal of increasing the working titre of the antiserum and also improving the stability of the coated latex. The LAIA was then applied to the analysis of meat extracts. As the elution of SMZ from the SPE microcolumn requires use of an organic solvent (methanol), it was necessary to establish that this would not affect the performance of the antibody in the LAIA; it was established that the assay was not affected by the presence of up to 40% methanol in the sample extract. A series of sample extracts of pork were prepared by the MSPD/SPE method. The extracts were spiked with various concentrations of SMZ, ranging from 25 to 250 ng/ml and were analysed "blind" by the LAIA method. These studies showed that further clean-up of the pork

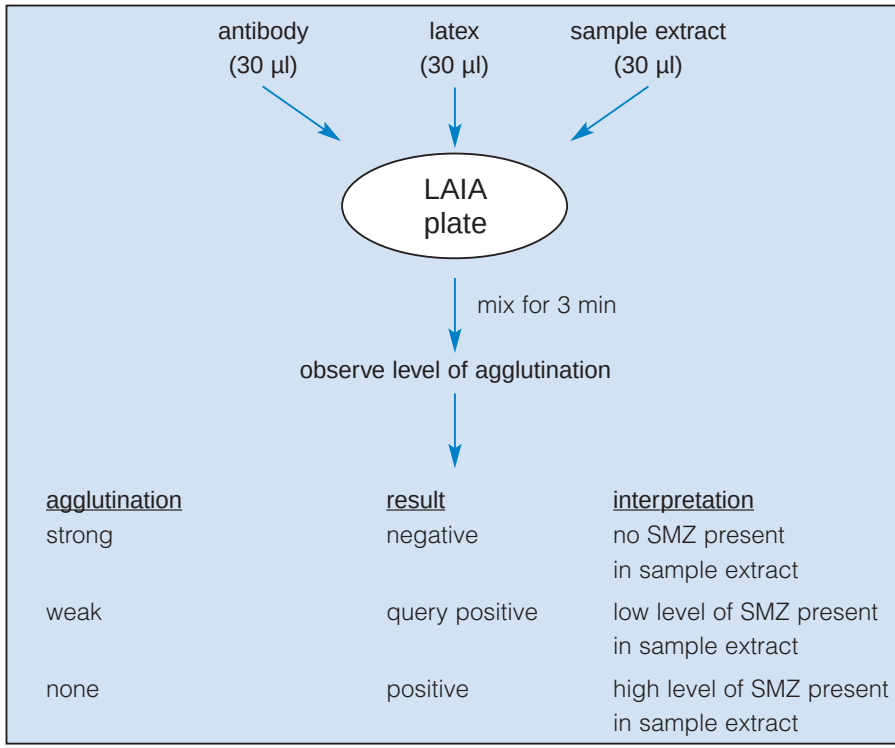

Figure 4: Screening of pork muscle samples using the LAIA procedure.

extracts, in the form of a filtration step post-SPE to reduce the cloudiness of the extracts, was required to maximise the agglutination reaction.

DEVELOPMENT OF A PROTOTYPE KIT FOR SMZ

The various components of the method were combined to form the test kit, which was evaluated in an inter-laboratory "blind" trial. Samples containing various concentrations of SMZ were extracted and analysed according to the test procedure. The test kit correctly screened samples for SMZ at levels above and below the MRL of 100 ppb (see Table 1).

A prototype kit containing mainly disposable items was prepared for evaluation. The kit consisted of glass mortars and pestles (for the MSPD procedure), custom-built racks to hold the prepared MSPD cartridges and the SPE cartridges, C18 sorbent, silica SPE cartridges and assorted glass and plastic components. The kit included, also, glass slides for the agglutination reaction and samples of latex particles coated with SMZ-bovine serum albumin conjugate, and rabbit anti-SMZ antiserum. Each kit was sufficient to screen eight pork samples for SMZ. The kit was evaluated at the collaborating laboratories as part of an inter-laboratory blind trial. Eight samples containing a range of SMZ (0-300 ppb), i.e. above and below the MRL of 100 ppb, were evaluated. Results

Table 1: Evaluation of the new test kit method for detecting sulphamethazine in a laboratory "blind" trial.

Sample description	SMZ* (ppb)	Agglutination observed	Sample classification
Containing no SMZ	0	Strong	Negative
Containing SMZ below the MRL	53	Weak	Query Positive
Containing SMZ at the MRL (approx.)	113	None	Positive
Containing SMZ above the MRL	252	None	Positive

*SMZ content determined by HPLC analysis

showed that all samples that were free of SMZ or which contained residues at or greater than the MRL were correctly classified as "negative" or "positive", respectively. Samples containing SMZ residues below the MRL were classified as "negative", "query positive" or "positive". Results were confirmed by high performance liquid chromatography (HPLC) analysis. Good comparability in use of the prototype kit between the two laboratories was achieved (Table 2).

In-industry evaluation of the prototype kit

Two pork processing plants were recruited for an industry evaluation of the kit (O'Keeffe et. al., 2000). A protocol of the operating procedure and the kit components were supplied to each participating laboratory. The kit test procedure required comparison of the response for the test pork extracts with

Table 2: Evaluation of the new test kit method for sulphamethazine in an inter-laboratory 'blind' trial. The maximum residue limit (MRL) for sulphamethazine in meat is 100 ppb.

SMZ* (ppb)	Agglutination Observed		Sample classification
	Laboratory 1	Laboratory 2	
0	Strong	Strong	Negative
0	Strong	Strong	Negative
0	Strong	Strong	Negative
26	Weak	Strong	Query positive/Negative
55	None	Weak/None	Query positive/Positive
113	None	None	Positive
179	None	None	Positive
270	None	None	Positive

*SMZ content determined by HPLC analysis

the responses for negative and positive reference control sample extracts, which exhibit strong and no agglutination, respectively. The test sample extracts are classified as "negative", "query positive" or "positive", represented by responses of strong, weak and no agglutination, respectively.

The method was demonstrated to the industry tester using eight pork samples containing various concentrations of SMZ. Following the demonstration, each industry tester was supplied with a further eight samples for a trial evaluation of the kit, with the tester carrying out the procedure independently, but under observation. Finally, on a separate occasion, each industry tester was supplied with a further eight samples for a "blind" evaluation of the kit, to be carried out totally independently. Results showed that all samples containing no SMZ were correctly classified as negative by both industry testers (Table 3). All

Table 3: Results of an industry evaluation of the new test kit for sulphamethazine using incurred pork muscle samples. The maximum residue limit (MRL) for sulphamethazine in meat is 100 ppb.

	Agglutination Observed		
SMZ* (ppb)	Industry tester 1	Industry tester 2	Sample classification
0	Strong	Strong	Negative
0	Strong	Strong	Negative
0	Strong	Strong	Negative
26	Weak	Weak	Query positive
55	Strong	None	Negative/Positive
113	Weak	None	Query positive/Positive
179	None	None	Positive
270	None	None	Positive

*SMZ content determined by HPLC analysis

samples contining SMZ at greater than the MRL of 100 ppb were classified as positive or query positive by both testers. Some problems occurred in classification of samples containing SMZ at levels below the MRL. It was concluded that more training, particularly in the detection step, which is quite subjective, would result in clearer results.

Sol particle immunoassay

A lateral flow membrane-based assay, also known as a sol particle immunoassay (SPIA), for SMZ (obtained from Euro-Diagnostica B.V., Arnhem, The Netherlands) was evaluated as an alternative to the LAIA system.

The SPIA consists of collodial particles conjugated to SMZ-specific antibody (the "label" for the immunoassay) and a lateral-flow membrane device serves as the basis for separation and focusing of the antibody-bound SMZ. The sample extract is loaded onto the sample zone at one end of the test strip and any SMZ present in the sample extract is bound by colloid-antibody and migrates along the lateral flow membrane. A capture zone, impregnated with SMZ bound to protein, captures any free colloid-antibody as a narrow band which is visible to the eye. Therefore, sample extracts free of SMZ will result in free colloid-antibody which will give a colour reaction at the capture zone. Alternatively, residue-positive sample extracts, which result in binding of SMZ to the colloid-antibody, are identified by the absence of a colour reaction at the capture zone.

Pork samples containing various levels of SMZ were extracted by the MSPD/SPE method and were analysed by the SPIA procedure (Figure 5). The development of a line in the "test" zone 15 minutes after sample application is indicative of a negative sample while the absence of a line in the "test" zone indicates a positive sample.

Initial experiments on both fortified and incurred pork showed that the assay was capable of differentiating between negative and positive samples at levels of SMZ below the MRL. The system was further evaluated by the analysis of eight sample extracts by two analysts independently in the laboratory. As outlined in Table 4, good comparability of results was obtained between the analysts.

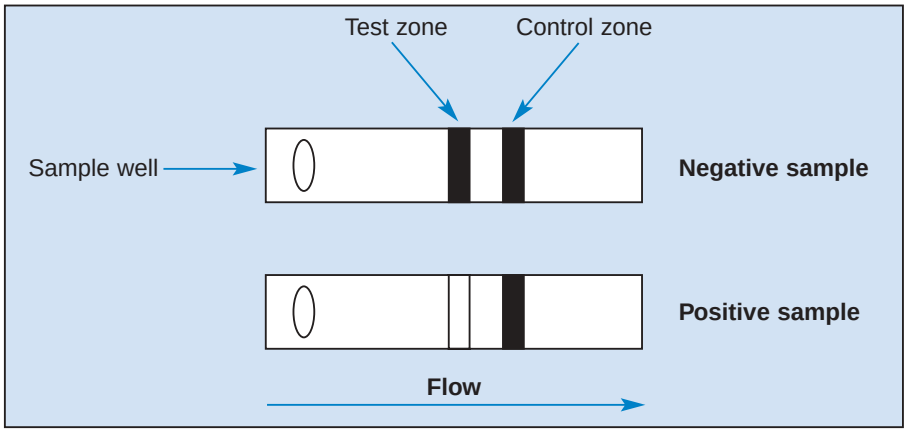

Figure 5: Screening of pork samples using the SPIA procedure.

Table 4: Results of laboratory evaluation of the SPIA method using incurred pork samples.

SMZ* (ppb)	Appearance of line in test zone		Sample classification
	Analyst 1	Analyst 2	
0	+	+	Negative
0	+	+	Negative
0	+	+	Negative
28	+/-	-	Query positive/Positive
38	+/-	-	Query positive/Positive
97	-	-	Positive
164	-	-	Positive
227	-	-	Positive

*SMZ content determined by HPLC analysis.

Eight sample extracts were also sent to a pork industry tester, who had previously been trained in the MSPD/SPE/LAIA kit method. The results obtained (Table 5) suggest that the SPIA is capable of detecting all samples containing SMZ residues, even at concentrations much lower than the MRL.

Table 5: Results of an industry evaluation of the SPIA method using incurred pork samples incurred with SMZ.

SMZ* (ppb)	Appearance of line in test zone	Sample classification
0	+/-	Query positive
0	+/-	Query positive
0	+	Negative
28	-	Positive
38	-	Positive
97	-	Positive
164	-	Positive
227	-	Positive

*SMZ content determined by HPLC analysis.

CONCLUSIONS

- A method incorporating matrix solid phase dispersion, solid phase extraction and a latex agglutination inhibition assay was applied to the extraction and determination of sulphamethazine in pork muscle. The method was shown to be sensitive and robust.
- A prototype kit was assembled from the developed procedure for the analysis of sulphamethazine in pork tissue and was shown to be capable of differentiating between residue-free samples and samples containing sulphamethazine at the maximum residue limit of 100 parts per billion.
- The prototype kit was evaluated in industry and exhibited potential as a possible screening method for "on-site" determination of sulphamethazine in pork carcasses.
- A sol particle immunoassay was applied, as an alternative system to the latex agglutination inhibition assay, to reduce the possible subjectiveness in interpreting the latex agglutination.
- This project demonstrates the capability for developing robust testing kits, based on efficient extraction and immunochemical residue determination, which may be applied to screening of carcasses for the presence of antibiotic residues.

RECOMMENDATIONS TO INDUSTRY

The research described in this report is aimed at developing kit methods for use by the food industry, food quality inspectors and laboratories to test for the presence of antimicrobial residues in food. Within Europe, monitoring for residues of veterinary drugs in foods of animal origin is carried out under EC Directive 96/23 which covers both official testing and also a requirement for "self-monitoring" by the food industry. Apart from these legal requirements, food companies may wish to undertake their own specific controls on food products.

In the area of antimicrobial testing, there are a range of test options. Inhibitory substance testing, which is a broad screening approach to identify the presence of antimicrobial residues, may be applied in the form of agar plate tests (Four-Plate, One-Plate) for meat or in the form of tube tests for meat (e.g. Premi®test) and milk (e.g. Delvotest, AIM 96, BRT, Valio T 101). More specific tests, for particular families of antimicrobials, are based on enzymatic (e.g. Penzym) and immunological (e.g. Charm II, LacTek) tests. Additionally, there are a range of enzyme immunoassay (ELISA) tests from companies such as Randox, Biopharm and Euro-Diagnostica for specific antimicrobials. Dip-stick or card tests have been developed, such as Ez-Screen and the Euro-Diagnostica SMZ-SPIA, which was used in the research reported here.

The food industry have available to them a range of test systems which can be applied to ensure the quality of their food products. Selection of an appropriate test system depends on a range of factors such as whether broad screening or testing for specific antimicrobial residues is required and what standard of laboratory facilities and technical expertise are available.

[Note: Commercial test products mentioned are given as examples only and are not necessarily endorsed or recommended by the authors.]

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