Chapter V

Analysis of Antibiotic and Drug Residues in Agrifoods and Seafoods

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A. INTRODUCTION

Regulatory Overview

In United States the US Food and Drug Administration's Centre for Veterinary Medicine, Department of Health and Human Services, has the authority to regulate veterinary drug products in foods. The US Federal Food, Drug, and Cosmetic Act (FFDCA) covers food safety, medical devices, and other public health matters. Section 512 of the FFDCA deals with general safety provisions for new animal drugs while Section 409 and 706, respectively, cover safety aspects of food additives and colour additives. The code of Federal Regulation (CFR) subpart E relates directly to the safety of foods consumed by humans.

In Canada the Bureau of Veterinary Drugs, Health Drugs, Health Protection Branch, Federal Department of Health, is responsible for regulating use of veterinary drugs in food-producing animals. This process closely resembles the registration process in US as provided under a harmonisation initiatives of US-Canada Free Trade Agreement.

Regulatory authorities in US and Canada have established maximum residue limits (MRLs) for edible tissues. Unlike Canada, US has procedures for establishing an MRL for non-edible tissues when this tissues is the best target tissue for marker residue. The most common target tissue is liver or kidney.

Although the regulatory authority approving substances in US lies with FDA's Centre for Veterinary Medicine (CVM), the regulatory authority for determining compliance of animal-derived foods is the US Department of Agriculture, Food Safety and Inspection Services (FSIS). The residue control programs established by FSIS include the National Residue Program (NRP) major component of which includes antibiotic residue testing.

In Canada residue monitoring of drug residues in edible tissues is the function of Canadian Meat Inspection system of the Food Production and Inspection system of the Food Production and Inspection Branch, Federal Department of Agriculture and Agrifoods. Monitoring for antibiotic residues in cultured finfish is the responsibility of the Inspection Service Branch, Federal Department of Fisheries and Oceans.

There are less than 30 antimicrobial drugs approved by FDA for use in food-producing animals in the US. The tolerance of antimicrobial residues in foods permitted by the FDA ranges from approximately 0 - 10 ppm.

Although the average intake of meat has increased after World War II, the consumption of fish in Japan remains high when compared to other countries[1]. The different categories of feed additives in use in Japan include antioxidants, and anti-fungal agents (to prevent spoilage of feed) as well as 7 amino acids, 28 vitamins, and 32 minerals (used as dietary supplements). A separate group of 7 synthetics antibacterial with 21 antibiotics is used as feed additives to improve the efficiency of feeds[1].

In Japan use of antibiotics as feed additives and veterinary medicinal drugs are regulated by the Pharmaceutical Affairs Law and the Law Concerning Safety Assurance and Quality Improvement of Food. Another essential law is the Japanese Food Sanitation Law which states that no food should contain any antibiotic or synthetic antibacterial substances in meat, eggs, fish, shellfish, milk and dairy products.

In the European Union (EU) veterinary medicinal products are regulated as two distinct groups. The conventional therapeutic drugs, including those for prophylactic treatments, vaccines, and immunological products, are regulated under the Veterinary Medicine Directives 85/851/EEC and 85/852/EEC. The growth promoting antibiotics, the peptides, carbodox, coccidiostats, and olaquindox are regulated under Directive 70/524/EEC. Recent development in EU is the establishment of European Medicine Evaluation Agency (EMEA) based in United Kingdom. This agency authorises the use of drugs and many decisions are binding on Member States. Special EU legislation deals with the establishment of MRLs and are established by the Committee for Veterinary Medicinal Products (CVMP) through its Working Group on Safety of Residues.

Screening Methods

In both Canada and the US, testing programs based on evaluating animals at slaughter houses for the presence of antibiotic residues utilise one of the two commercially available microbial growth inhibition tests. The program uses either the Swab Test on Premises (STOP)[2] or the Calf Antibiotic and Sulfa Test (CAST)[3]. Both tests were originally developed by scientists at the USDA-FSIS. These tests although similar in principle use different growth media and different test organisms, providing a different range of test sensitivities to various antibiotic residues that may be present in samples[4].

In Canada, STOP and CAST test kits are used in random surveys of slaughter house animals to test for the prevalence of violative antibiotic residues. The suspect carcasses are detained pending on laboratory testing of kidney and muscle tissues.

The FSIS Microbiology Division has recently developed a new antimicrobial screening test designed to detect the presence of antibiotic and sulfonamide residues in animal tissues. The new test, Fast Antimicrobial Screen Test (FAST) was designed to read an in-plant test result in a shorter time than either STOP or CAST. FAST is currently implemented in calf slaughtering plants in the US.

Other screening tests used in US and Canada include screening of eggs, egg-products, and dairy products using Brilliant Black Reduction Test (BBR Test), the Charm II tests, Delvo Tests, and LacTek Tests.

In Japan a method based on microbiological assay[5] is used for detecting and classifying antibacterial residues. This method applies a filter paper disc method using Bacillus subtilis (ATCC 6637), Bacillus cereus var mycoides (ATCC 11778), and Micrococcus luteus (ATCC 9341) in identifying antibacterial residues in fractions of samples obtained by chemical separation. Fractions A, B, and C contained different antibiotics that give different growth inhibition patterns which allow their rapid identification and classification. The purpose of a screening is to quickly determine whether or not an analyte is present at or near the level of concern in the target sample.

Amongst member states of European Community the Four Plate Test[6] is very popular. For this method, also known as Frontier Post Test, uses four plates with three different organisms (Bacillus subtilis, two different species, and Micrococcus luteus). The organisms are grown at different pH conditions such that they respond differently to different antibiotics. Two discs of meat or offal removed from the sample with sterile cork borer are placed on the plates in duplicates. Discs of standard penicillin, streptomycin, sulfadimidine, and erythromycin are used as controls to indicate that the test has been successfully performed.

A test is positive when an annular zone greater than 2 mm appears around the discs taken from the food samples. The purpose of running four plates is to attempt to obtain some indication of the class of antibiotic that may be present. From the size and the pattern of the annular zones of inhibition it is suggested that some guidance can be obtained regarding the class of antibiotic in question.

Chemical Method

The use of chemical methods for antibiotic residues is limited because microbial inhibition methods are commonly used for regulatory purposes. Application of high-performance liquid chromatography methods for antimicrobials have been described[7-9]. The current range of chemical analysis techniques such HPLC, GC-MS, and HPLC-MS are powerful and useful for drug residue analysis; however complete facilities for analysis are expensive to set up. Currently HPLC methods are used for official analysis of synthetic antimicrobials.

There are two chemical methods for penicillin used by the EU Member States. One is the GC method of Miltschen and Petz[10] and the other is HPLC method of Boison et al[11].

The main chemical methods used for tetracyclines (tetracycline, chlortetracycline, and oxytetracyclin) are those of Ikai et al[12], Farrington et al[13], and Blanchflower[14].

Aminoglycosides pose problems in chemical analysis. Their solubility properties and their lack of distinctive chromaphores offer obstacle to an analyst. However, a very

recent publication of a method for streptomycin and dihydrostreptomycin[15] has appeared. A method for gentamycin[16] has been published in the EU Manual[17] that may be useful for neomycin.

Chemical methods for chloramphenicol available in EU utilise either GC (with or without coupled MS) or HPLC-GC methods[18,19]. There are several published methods for chloramphenicol[20,21].

Very little attention is being paid to develop chemical methods for aminoglycosides, macrolides, peptides antibiotics and ionophores. For monensin TLC and HPLC methods are available[22, 23].

REFERENCES:

(1) Nakazawa, H.(1995). Overview of Antibiotics Used for Agriculture and Residual Analysis in Japan. In, Chemical Analysis for Antibiotics used in Agriculture. H. Oka, H. Nakazawa, K. Harada, and J. M. MacNeil, (eds). AOAC International. p31.

(2) Johnston et. al. (1981) J. Food Protect. 44: 828.

(3) United States Department of Agriculture, Performing the Calf Antibiotic and Sulfa Test. A Self-Instructional Guide. (Washington D. C.: United States Department of Agriculture, Food Safety and Inspection Service, Meat and Poultry Inspection Program, 1984)

(4) Korsrud, G.O., and J.D. MacNeil (1988).J. Food Protect. 51: 43.

(5) Jinbo et al.(1991) J. Food Hyg. Soc. Jpn.32: 86.

(6) Bogaerts, R and F. Wolf (1980) Fleischwirtsch 60: 672

(7) Moats, W.A.(1984) J. Chromatogr. 317: 311

(8) USDA, (1991) " Agricultural Uses of antibiotics ". ACS Symposium Series, No. 320. W. A. Moats (Editor). Washington D. C.: United States Department of Agriculture, Food Safety and Inspection Service.

(9) Moats. W. A.(1990) J. Assoc. Off. Anal. Chem. 73: 343.

(10) Meetschen, U. and M. Ptez.(1991) Z. Lebensmittel Unters Forsch. 193: 337.

(11) Boison et al. (1991) J. Assoc. Off. Anal. Chem. 74: 497.

(12) Ikai et al. (1987) J. Chromatogr. 411: 313

(13) Farrington et al.(1991) Food Addit. Contam. 8: 55

(14) Blanchflower, et al. (1989) Analyst 114: 55

(15) Gerhardt et a; (1994) J. Assoc. Off. Anal. Chem. 77: 334.

(16) Sur et al (1993) Anal . Chim. Acta. 275: 285.

(17) Heitzman, R.J.(editor) Residue in food Producing Animals and their Products: Reference Materials and Methods. "Veterinary Drug Residues" (Luxembourg: Office of Official Publication of the European Communities, L-2985, 1992)

(18) Arnold, D. and A. Somogyi (1985) J. Assoc. Of. Anal. Chem. 68: 984.

(19) van Ginkel et al (1990) Anal. Chim. Acta. 257: 61.

(20) CNEVA Laborataire des Medicaments Veterinaire. Fougers, Framce, Doc. UCM/90/04.

(21) Keukens et al (1986). J. Chromatogr. 352: 445.

(22) Okada et al. (1980). J. Food Hyg. Soc. (Jpn) 21: 77 .

(23) Tihova, D. and V. Peneva (1982). Vet. Med. Nauki. 19: 52.

B. DETECTION OF DRUG AND ANTIBIOTIC RESIDUES USING RAPID KITS

Scope:

Antibiotics are used frequently in modern agriculture and aquaculture practices, both as additives to feed and water to enhance weight gain, improve feed efficiency and to prevent diseases; in higher dosages they are useful in treating specific diseases in terrestrial as well as aquatic animals. Consequences of such usage is contamination not only of food products but also the environment. Thus, routine inspection of food products has become an important function of public health agencies and regulatory authorities all around the world.

Not only has there been a great leap in the production of food globally but also international food trade has increased considerably. This has generated growing need for more and rapid testing methods. Public health and regulatory agencies as well as producers need help to cope up with increased volume of testing; they are looking for rapid and more cost-effective mechanisms to face the problem of food testing and meet the consumer demand for products free of antibiotic or chemical residues.

Biological assays introduced in early 1970s had appropriate properties required to meet high-volume testing. These microbiological assays, however, failed to provide the desired specificity in the detection of specific antibiotics. Today both microbiological and immunological assays are used with improved specificity and sensitivity.

Principle:

a. Microbiological Inhibition Tests

Microbiological assays involve growth (either on solid agar or liquid broth medium) and are based on definable parameters which characterise response of test organisms in the presence of antibiotics. The microbial response to a series of standard antibiotic concentrations with specific strain of a test organism is used to determine the levels of an antibiotic in an unknown sample.

Agar diffusion tests are conducted by inoculating a nutrient agar with specific test organism and applying a solution containing the drug residue to the agar surface in more than one way. The growth medium is then incubated for a specific period of time at a specified temperature.

In biological assays the response of the test organism is determined by measuring the zone of inhibition (ZI) formed in plates in the presence of antimicrobials. Tests that include measurement of ZI include Live Animal Swab Test (LAST), Calf Antibiotic and Sulfa Test (CAST), Swab Test on Premises (STOP), and Bacillus stearothermophilus disc assay (BSDA).

Another method to test inhibition of microbes is by incorporating redox indicators in the test medium. Examples of such test include Brilliant Black Reduction Test (BBR), Arla Micro Test, and Rapid Antibiotic Test (LUMAC). A redox indicator

like Brilliant Black is purple in its oxidised form and yellow when reduced. Thus organisms growing in the absence of inhibitors will cause a colour change from purple to yellow. In the presence of antimicrobials the lack of metabolism will result in no colour change (negative test).

Inhibition tests which involve colour changes are dependent on acid-base indicators. For example bromocresol purple yields yellow colour at pH5.2 and a purple colour at pH 6.8. Microbial metabolism in the presence of fermentable sugars added to the medium results in acid production causing the pH to drop. This change results in alteration of colour of the medium. Tests that make use of colour change of the medium include Charm Farm Test, Fast Antibiotic Screen Test (FAST), Charm Inhibition Assay (CIA), Delvotest and Delvotest SP, BSDA, and Valio T101.

b. Immunological Assays

The principles of immunoassays are similar regardless of whether isotopic or non-isotopic techniques are used. Radioimmunoassays are based on the competition between a labelled (with radioactive or non-radioactive) analyte (antibiotic or drug) for a binding site on an antibody. The presence of a large quantities of unlabeled analyte in a sample results in less radioactivity bound to the antiserum. A comparison of the ratio of the bound to the free labelled analyte with that obtained from a series of standards permits to quantitate the analyte in an unknown sample.

Enzyme immunoassays (EIAs) are non-isotopic immunoassays that use enzymes as labels. Enzyme-linked immunosorbent assays (ELISA) include separation of an enzyme-labelled antigen or antibody complex from a free enzyme-labelled antigen or antibody. ELISA tests have been developed in which wells or microtiter polystyrene wells or plates or membranes such as polystyrene , nylon, and nitrocellulose are used as solid phase to immobilise either antibodies or antigens. These membrane are coated onto single tubes, cups, dipsticks, or discs upon which tests are conducted. Commercially available ELISA test kits for detecting antibiotic residues in food and biological fluids include Cite Probe (Idexx), LacTek, Beta-Lactum, Lactek Ceftiofur, LacTek Tetracycline (Idetek), Penyme/Penzyme III, Signal (SmithKline Beecham), Ridascreen Tetracyline (R-Biopham Gmbh), SingleStep Block, and SingleStep ELISA (Environ. Diagnostic).

In these tests antibodies against specific drugs are immobilised on to a solid phase. A specific volume of a sample containing the free drug of interest is added to the immobilised antibodies followed by the addition of an enzyme conjugate. The mixture is allowed to incubate for a specified time during which the free drug competes with the enzyme-labelled drug (hapten) for the antibody binding sites. After incubation period, any unbound free drug or enzyme-bound drug are washed away; an enzyme substrate solution is added and the test system is usually chosen so that it is colourless initially but yields a green or blue coloured products when acted upon by the enzyme. The blue or green colour development indicates that there was no significant amounts of antigen (drug residue) in the test sample

(negative test). On the other hand no colour formation indicates the presence of the residue (positive test).

C. MICROBIAL INHIBITION RAPID TESTS (STOP, CAST, AND LAST)

Scope:

These tests kits are manufactured by Iditek Inc., Burlington, North Carolina, USA., and are used for screening animals for the presence of antibiotics and sulfa residues. The sample containing drug residues may represent animal tissues (kidney, muscle, liver), animal fluids (urine, serum, or tissue fluids) or antimicrobials extracted from animal/fish tissues, grains, and animal/fish feed.

Principle:

The scientific basis of these tests includes inhibition of test organisms in the presence of antimicrobial substances. An agar plate is uniformly seeded with spore suspension of Bacillus subtilis (STOP, LAST) and Bacillus megaterium (CAST). As the spores germinate, a uniform lawn of bacterial colonies grow on the surface of the agar.

A cotton swab saturated with a sample is positioned on the agar surface. If antimicrobials are present in the sample, these will diffuse out into the agar layer and prevent or inhibit the growth of bacterial colonies in an area around the swab. A clear zone of inhibition (ZI) around the swab indicates the presence of antimicrobial substance (positive test). A uniform lawn of bacterial colonies around the swab head reveals the opposite (negative test).

Sample Preparation:

These tests are primarily intended for testing poultry and cattle kidney tissues (carcasses) for the presence of antibiotic residues. However, the tests work equally well using liquid samples containing residues extracted from animal, or fish tissues and animal feed samples.

Materials:

Most of the materials needed to perform the tests are included in the kits. Agar plate: Ten plates per kit.

Spore suspensions: Spore suspensions of test organisms for LAST, CAST, and STOP, 4 ml vial per kit (Bacillus subtilis for LAST and STOP; Bacillus megaterium for CAST).

Swabs: Sterile cotton swabs, 30 per kit.

Felt-tip marking pen: One per kit.

Forceps: One per kit.

Neomycin discs: Ten per kit

Plastic ruler: One 6 in ruler per kit.

Sample containers: Twenty containers per kit.

Note: Always keep agar plates, vials of spore suspensions, and neomycin discs in a refrigerator at all times except when in use. Materials not supplied but may be needed include, a knife or a pair of scissors, clean paper towels, and incubators, 44-45°C (for B. megaterium) and 27-29°C (for B. subtilis).

Procedure:

(a) Remove one agar plate and a vial of spore suspension from the refrigerator. Allow them to warm up to the room temperature.

(b) Select one of the test samples (urine, kidney, muscle tissue or liver, or liquid sample of a residue extracted from a test sample).

(c) For a kidney tissue sample make a smooth incision, using a clean knife, about 1-2 cm deep and two-thirds the length of the kidney. Follow the same procedure for a muscle or liver tissues.

(d) Select one sterile cotton-tipped swab and gently swab the incised surface with it. For liquid samples soak the swab. Ensure that the swab is completely saturated with the sample. Carefully and gently re-swab the incised surface or the muscle tissue.

(e) Using a marking pen, mark the sidewall of the agar plate with an "X". Rotate the plate so that the "X" mark is on the far side of the plate.

(f) Select an appropriate vial of the spore suspension and shake it vigorously to thoroughly mix the contents.

(g) Select one sterile cotton-tipped swab and dip it into the spore suspension to thoroughly saturate the tip.

(h) Withdraw the swab from the vial, recap the vial, and set it aside. Carefully hold the swab so it does not touch anything.

(i) Lightly swab back and forth across the width of the agar plate. Entire agar surface must be covered with the spore suspension.

(j) Using a pair of clean thumb forceps remove a neomycin disc and position it on the agar surface of the swabbed plate beside the "X" mark on the side wall of the

plate. Lightly touch the neomycin disc with the tip of the forceps to firmly seat the disc on the surface of the agar.

(k) Carefully break the swab stick about 1.5 cm below the saturated tip. Discard the swab stick. Position the saturate swab on the agar surface directly across from the neomycin disc. Gently press the swab with forceps to firmly seat it on the surface of the agar.

(l) Repeat Step 11 if additional samples have been prepared. Two swabs can be placed in one plate provided they are properly positioned and identified as shown in the illustration.

(m) Incubate the agar plates at the appropriate temperatures for at least hours but not more than 24 hours.

(n) Carefully examine the agar surface around the neomycin disc. The area around the disc that is clear of bacterial growth (zone of inhibition, ZI) indicates that the positive control is effective. Lack of ZI suggests that there is a possible problem with the material or the procedures.

(o) A ZI around the head of the swab indicates that antibiotics present in the saturated swab head have diffused through the agar and inhibited bacterial growth in the ZI.

(p) The ZI around the swab head indicates that the test is positive. The absence of ZI around the head of the swab indicates that the test is negative.

(q) For interpretation of results see the illustration.

Interpretation of Results:

A. ANTIBIOTIC NEGATIVE B. ANTIBIOTIC POSITIVE C. TEST INCONCLUSIVE

		A. Antibiotic Negative	B. Antibiotic Positive	C. Test Inconclusive
STOP	IF YOU HAVE...	Opaque bacterial growth right up to the swab tips	Clear zones around swab tips	Opaque bacterial growth right up to the swab tips
STOP	AND YOU HAVE...	A clear zone around the Neomycin disc 20 - 25mm	Clear zone around Neomycin disc between 20 - 25mm	Clear zone around Neomycin disc that is less than 20mm
STOP	THEN TAKE THIS ACTION...	Animal is ready for market	Animal has antibiotic residues	Rerun test
LAST	IF YOU HAVE...	Opaque bacterial growth right up to the swab tips	Clear zones around swab tips	Opaque bacterial growth right up to swab tips
LAST	AND YOU HAVE...	A clear zone around the Neomycin disc 16 - 24mm	Clear zone around Neomycin disc between 16 - 24mm	Clear zone around Neomycin disc that is less than 16mm
LAST	THEN TAKE THIS ACTION...	Animal is ready for market	Animal has antibiotic residues. Retest in 2 to 3 days	Rerun test
CAST	IF YOU HAVE...	Opaque bacterial growth right up to the swab tips	Clear zones around swab tips	Opaque bacterial growth right up to swab tips
CAST	AND YOU HAVE...	A clear zone around the Neomycin disc 26 - 29mm	Clear zone around Neomycin disc (N5) between 26 - 29mm	Clear zone around Neomycin disc (N5) that is less than 24mm
CAST	THEN TAKE THIS ACTION...	Animal is ready for market	Animal has antibiotic residues. Retest in 2 to 3 days	Rerun test

REFERENCES

1. Environmental diagnostics, Inc. Performing the Live Animal Swab Test (Burlington, NC: Environment Diagnostics Inc. 1979).

2. United States Department of Agriculture. Performing the Calf Antibiotic and Sulfa Test (Washington D. C.: United States Department of Agriculture, Food Safety an Inspection Services, Meat and Poultry Program, 1984).

3. Johnson, R.W. and et al (1981) J. Food Protect. 44: 828.

4. Association of Official Analytical Chemists, Official Methods of Analysis, 15th ed.(Arlington, VA: AOAC,1990) Sections 982.16A- 982.16D.

5. Richardson, G.H.(1985). Standard Methods of Analysis, 15th. ed., (Washington D. C.: American Public Health Association, 1985).

6. Thaker, N.H. and et al (1993). Fast Antimicrobial Screen Test (FAST). In-Plant Study. (Washington D. C.: United States Department of Agriculture, food Safety and Inspection Services, 1993).

D. DETECTION OF ANTIMICROBIALS USING IMMUNOLOGICAL METHODS

1. Singlestep Block Sulfamethazine (SMZ) and Sulfadimethoxine (SDM)

Scope:

These rapid tests are for determining the presence of SMZ and SDM extracted from feed, tissues, serum, milk, or urine with methanol/water or dilution buffer, clarified by filtration and diluted in dilution buffer provided with the kits.

Principle:

These tests are based on solid phase competitive immunoassays. In these tests SMZ and SDZ are coupled to an enzyme. this enzyme-bound drug competes with the free drug in the test sample for the antibody binding site. The presence of significant levels of the drug residue in the sample prevents the enzyme-bound drug from binding to antibody coated in the reaction sites and is washed out. Upon the addition of the substrate the enzyme (if bound to the antibody) acts on the substrate to give a blue coloured product. The presence of SMZ and SDZ in the test sample is indicated by the absence of the blue colour.

Materials:

Each kit contains:

(1) Foil wrapped testing devices. Each device contains 4 reaction sites.
(2) One red capped dropper bottle of Sulfamethazine enzyme.
(3) One green capped dropper bottle of Negative control.
(4) One yellow capped dropper bottle of Substrate A
(5) One blue capped dropper bottle of Substrate B.
(6) One white capped dropper bottle of wash solution.
(7) One bottle of dilution buffer.

Materials required but not included in the kits:

(1) Blender for grinding and mixing grain, feed, and nut, samples.
(2) Methanol/water solution (80:20), 100 ml per grain sample.
(3) Various size pipettes capable of transferring from 100 to 1000 μl volumes.
(4) Timer.
(5) Test tube or a small container to prepare solutions.

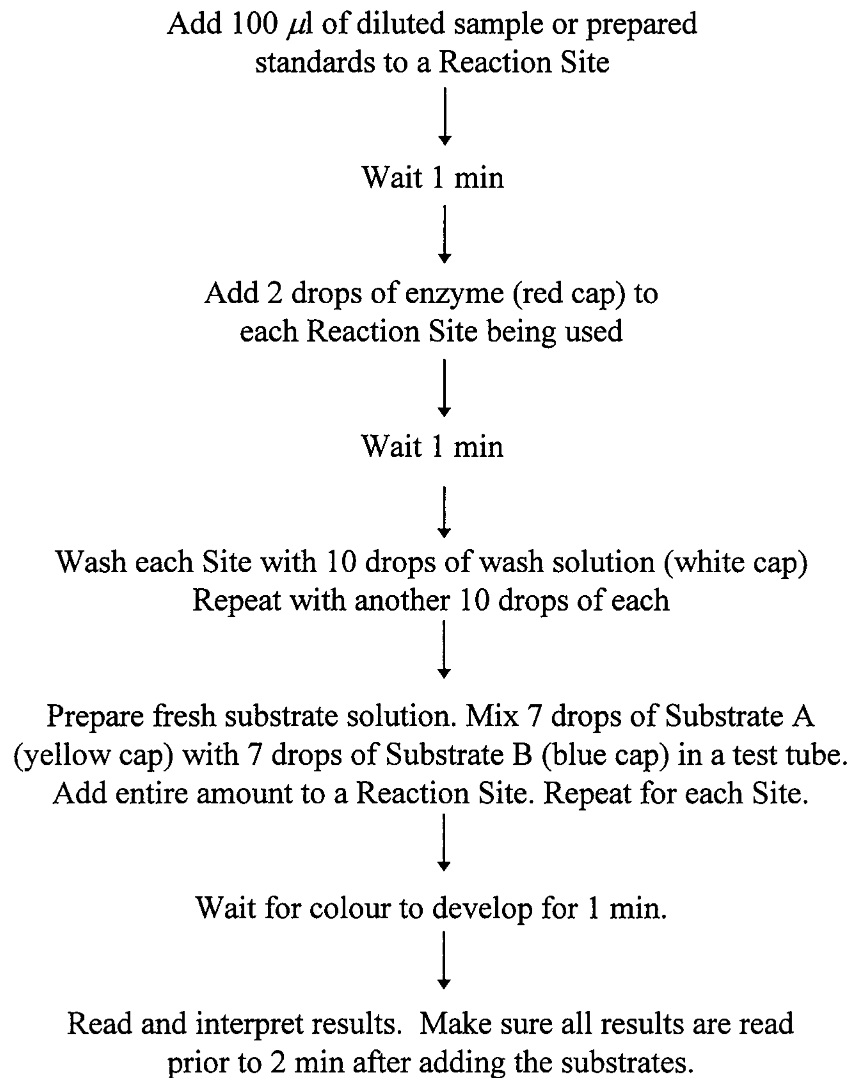
Preparation Of Standard Solutions Of Analytes:

(a) Dissolve separately 1 mg equivalent of SDM or SDZ (sodium salts) in 1 ml of methanol to prepare a 1 mg/ml solution. SDM and SDZ are available from Sigma Chemical Co., catalogue No. S-7385 and S-5637.
(b) Perform serial dilution in dilution buffer to attain the required level standard concentration.

Sample Preparation:

Follow the instructions provided with the kits.

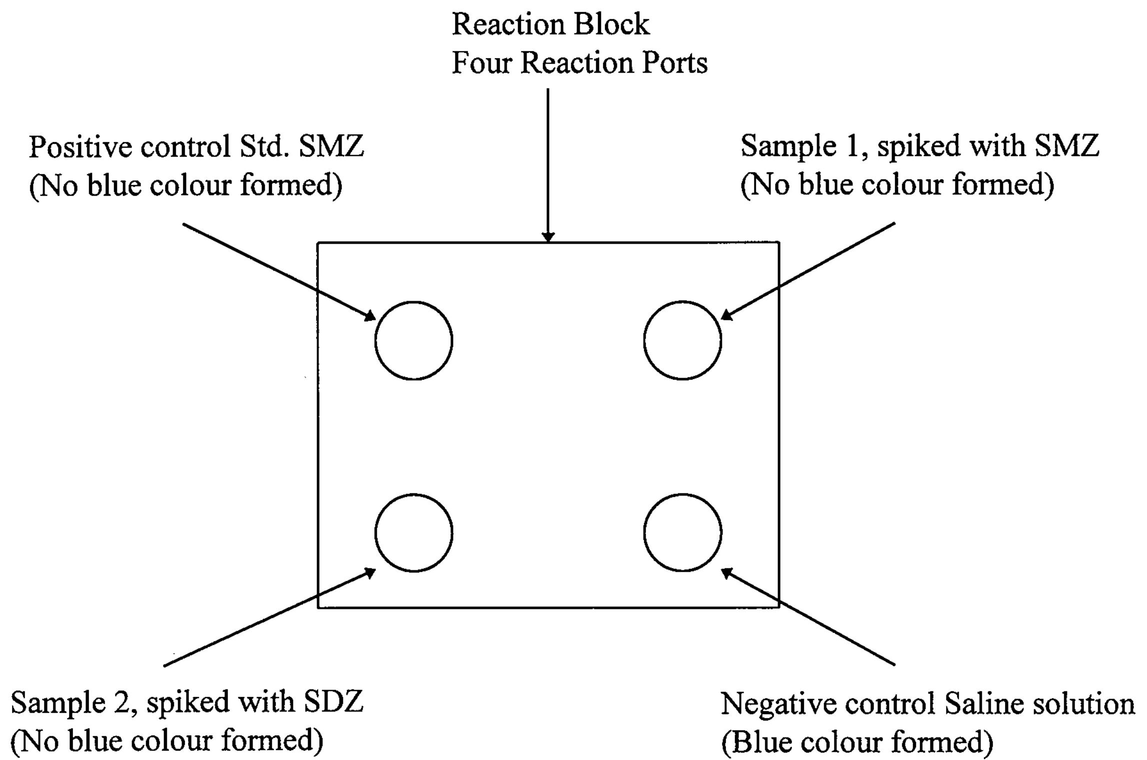
Procedures:



Interpretation of Results:

Blue colour indicates negative test (no residue in a sample) while no colour indicates positive test (presence of residue in a sample).

FOUR REACTION SITES ON A REACTION BLOCK



Sketch of A Testing Device (Reaction Block)
Illustrating the Positioning of the Reaction Sites

2. EZ-SCREEN Sulfamethazine and Sulfadimethoxine (QUICK CARD TEST)

Scope :

The tests are qualitative enzyme immunoassays for the detection of sulfamethazine or sulfadimethoxine in urine, serum, extracted samples of meat, fish, or feed. This screening procedure is intended to serve as an indicator of the presence of these residue levels that may be violative as defined by the USDA-FSIS's National Residue Program (NRP).

To assure the absence of potentially hazardous levels of the above drugs in edible foods, the Food and Drug Administration requires that all animals be drawn from the drugs for at least 15 days prior to slaughter. During this withdrawal period the residues are gradually cleared from the animal tissues and eliminated in the urine. Tolerance levels for uncooked edible tissues have been set at 0.1 ppm for cattle, swine, and poultry.

Principle:

The drug residues are extracted from samples with methanol/water, clarified by filtration and diluted in buffer provided with the kit or presented as a urine or serum sample and diluted in buffer provided with the kit. The diluted sample and negative control are added to the indicated QUICK-CARD test ports. Following absorption, enzyme conjugate is added to the test ports followed by a wash reagent and the substrate reagent. The analyte present in the sample competes with the enzyme-residue conjugate for the antibody bound to the QUICK-CARD. The presence of a significant level of a residue is indicated by the absence of colour at the test port.

Materials:

Each kit contains reagents or testing two samples and includes the following:

(1) QUICK-CARD: Two reaction sites per card are coated with rabbit antibody to a residue (sulfamethazine or sulfadimethoxine).
(2) ENZYME-RESIDUE CONJUGATE: The enzyme conjugate dropper tube contains a sealed ampule and reconstitution diluent. The glass ampule contains horseradish peroxidase conjugated to sulfamethazine or sulfadimethoxine. The lyophilized conjugate is reconstituted by breaking the glass ampule and gently shaking to mix.
(3) NEGATIVE CONTROL: The negative control dropper tube contains phosphate buffered saline solution.
(4) SUBSTRATE: The substrate dropper tube contains a sealed glass ampule and reconstitution diluent. The glass ampule contains substrate 4-chloro-1-naphthol and urea peroxide substrate. Reconstitute the tableted reagent by breaking the glass ampule and shaking to mix. The reconstituted reagent is stable for 8 hours at room temperature.
(5) DILUENT BUFFER: Two plastic screw-capped tubes containing 4.5 ml of phosphate buffered saline solution, pH 7.2.

(6) PIPPETTES: Four disposable pipettes. The pipettes deliver a drop having 50μl (0.05ml) when filled to a level of 1/2 inch.
(7) COTTON SWABS: Two cotton swabs.

Sample Preparation:

As per instructions provided with the Kits.

Test Procedure:

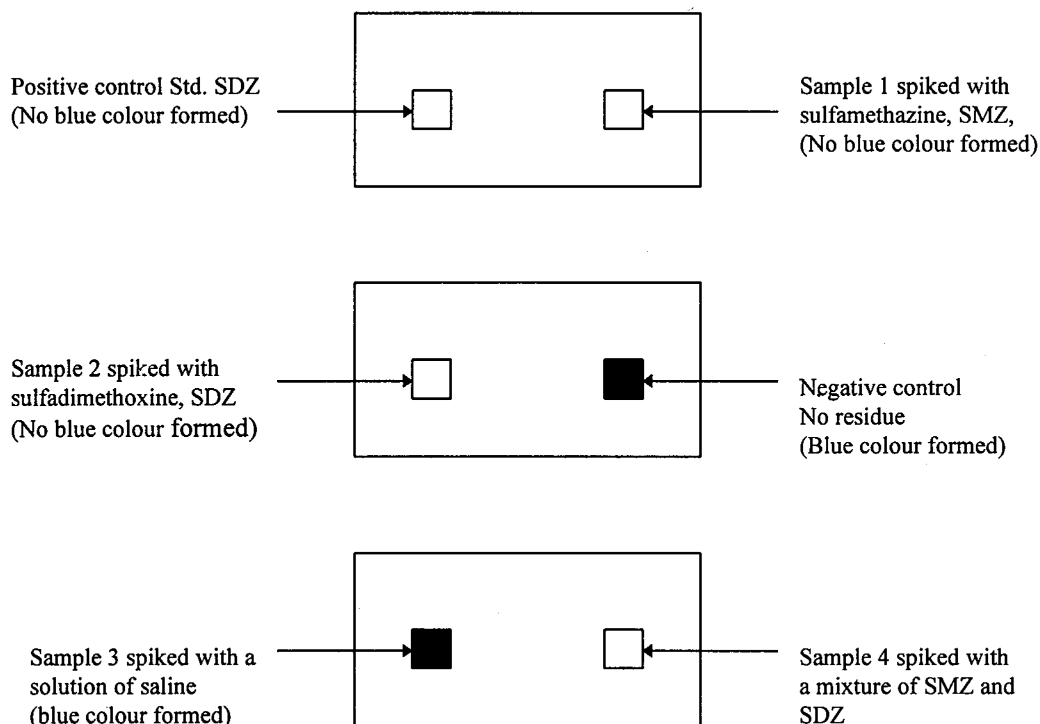
(a) Remove foil pouch from refrigerator and allow all reagents in the pouch to reach ambient temperature before opening.
(b) Prepare the Negative control by removing the plastic shrink seal from around the dropper cap.
(c) Prepare the Enzyme by squeezing the plastic dropper to break the inner glass ampule. Tilt the tube back and forth for approximately 20 seconds to re-hydrate and mix the contents. Remove the plastic shrink seal from around the dropper cap.
(d) Prepare the Substrate by squeezing the plastic dropper tube to break the inner glass ampule. Shake the tube vigorously. Remove the plastic shrink seal from around the dropper cap.
(e) Place the test card on a clean, flat surface and label it with the sample number and the date.
(f) When applying sample to the card, do not touch the sample pipette tip to the port. Hold the pipette so that the tip is about ½ inch above the port and allow the drop to fall freely.
(g) Apply one drop of Negative control to the "Control" port of the card.
(h) Apply one drop of a sample to the second port on the card. Allow the sample and control drops to absorb into the test ports before proceeding to the next step.
(i) Discard the first drop of Enzyme and then apply one drop to both of the ports on the card.
(j) Allow the Enzyme to absorb into the test ports before proceeding to the next step.
(k) Apply two drops of the Substrate to both the ports. Set a timer for 5 min.
(l) When the timer goes off, read the test results. If colour is visible in both the "Control" and "Sample" ports the sample is negative for sulfamethazine or sulfadimethoxine.

Interpretation of Results:

The sample is considered to be NEGATIVE for sulfamethazine or sulfadimethoxine when the "Sample" port develops readily detectable colour (light grey, grey-blue or blue) over the surface of the port.

The sample is considered to be POSITIVE for the residues when the "Sample" port fails to develop readily detectable colour (remains colourless).

Three QUICK CARDS used to test the presence of sulfamethazine and sulfadimethoxine in sample spiked with kinon concentration residues.



3. <u>RAPID SINGLESTEP ELISA for General Sulfa, Sulfadimethoxine and Sulfa methazine</u>

Scope:

These ELISA tests are solid phase immunoassays designed to rapidly detect and quantify a specific compound in a sample by means of a high affinity capture antibody. Liquid sample may represent extracts from animal tissues, seafoods, grains, or animal/fish feeds.

Principle:

Enzyme-conjugate (horseradish peroxidase is conjugated to a drug residue e.g. sulfadimethoxine or sulfamethazine) and antibody to this conjugate is prepared. The ELISA plate wells are coated with this antibody. A sample is added to the wells followed by and enzyme conjugate. During the incubation period, the conjugate competes with the drug residue in the sample for the binding sites on the antibody coated well. After a wash step to remove any unbound material, substrate is added for the final colour development. The colour intensity is inversely proportional to the amount of residue present in the sample. Those samples which contain the residue will inhibit binding of the enzyme conjugate to the antibody resulting in less colour than the negative control.

Materials:

The following materials are included in the kit:

(1) Antibody coated wells of a microtiter plate: The microtiter plate contains wells (twelve rows of 8 wells in each).
(2) Enzyme (lyophilized): Contains compound conjugated to the horseradish peroxidase enzyme.
(3) Enzyme diluent: Used to reconstitute lyophilized enzyme. Contains stabilised PBS.
(4) Sample and Standard Diluent: Contains 0.1% BSA in PBS.
(5) Standards (lyophilized): Reconstitute to 5 ml with sample and standard diluent.
(6) Wash solution: contains Tween-20 in PBS.
(7) Substrate: Contains 3,3,5,5- Tetramethylbenzidine.
(8) Stop solution: Contains 3N H_2SO_4.

Other materials required but not included in the kit:

(1) Micropipettes and tips.
(2) Disposable reagent troughs for pipetting reagent
(3) Glass culture tubes for dilutions.
(4) Microplate reader. Requires a 450 nm or 650 nm filter.

Assay Procedure:

Prior to performing test, allow one hour for all reagents to reach room temperature. Mix all reagents by gentle inversion.

(a) Reconstitute enzyme by adding 12 ml of enzyme diluent to the bottle of lyophilized enzyme. Let stand for 5 min, then mix by gentle inversion.
(b) To reconstitute standard, add 5 ml of sample and standard diluent to each bottle of lyophilized standard. Let stand for 5 min, then gently invert 10 times.
(c) Pipette 20 μl of sample and standard diluent (as negative control) into designated wells.
(d) Pipette 20 μl of the prepared standard(s) into adjacent wells.
(e) Pipette 20 μl of each prepared sample in the next available wells.
(f) Add 100 μl of the reconstituted enzyme to all wells. Mix by tapping the plate.
(g) Incubate the plate at room temperature for 10 min.
(h) Discard the solution from all the wells by inverting over absorbent paper. Pipette 400μl of wash solution in all the wells and discard by inverting the plate over an absorbent paper. Repeat this three times.
(i) Immediately add 150 μl of substrate to each well. Do not allow the wells to dry. Mix the reagents by tapping.
(j) Incubate at room temperature for 10 - 15 min or until a blue colour appears in the negative control wells.
(k) Add 150 μl of stop solution to all wells. Mix by tapping .
(l) Read results using a microplate reader at 450 nm wavelength.

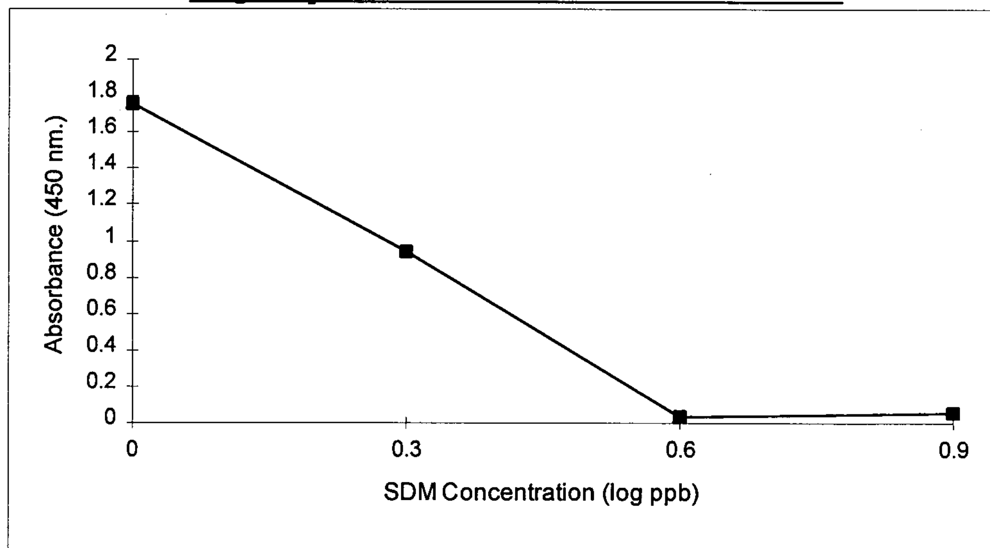
Interpretation of Results:

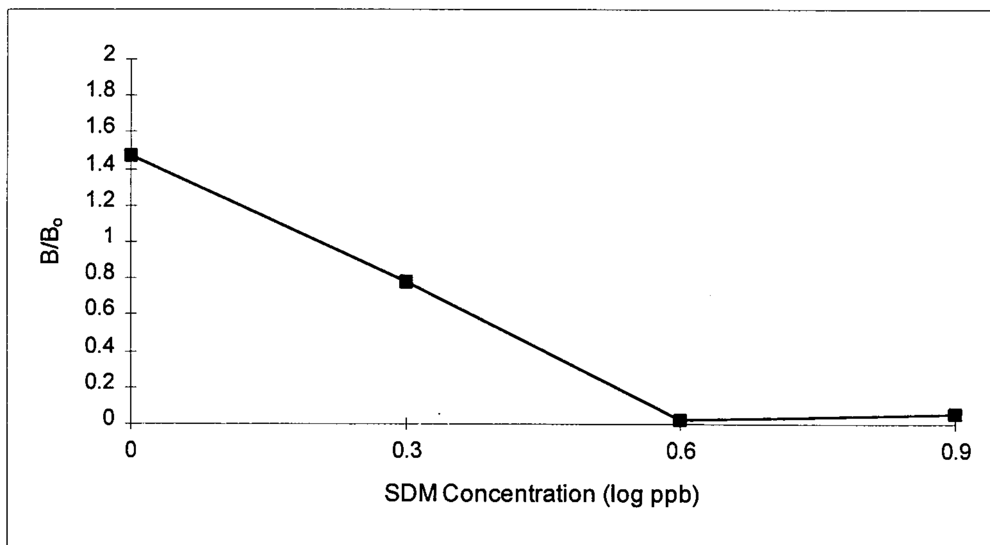
For the test to be valid, wells containing negative control samples must have a medium blue colour (unstopped) or a bright yellow colour (stopped).

Results:

Absorbency of the reactions in the microtiter plate wells was measured at 650nm using a plate reader. The results obtained are summarised.

Singlestep ELISA - Sulfadimethoxine Test Results





Std. SDM (ppb)	Absorbance (450 nm)	B/B_o
0	1.76	1.47
2	0.94	0.78
4	0.03	0.02
8	0.05	0.05

Computing Programs MicroELISA System Version 1.7 was used to read the microtiter plate.

E. RAPID ELISA TEST (RIDASCREEN) FOR SULFAMETHAZINE

Scope:

The test is competitive enzyme immunoassay for the quantitative analysis of sulfamethazine in milk and meat and for the qualitative analysis of sulfamethazine in meat. Sulfaemthazine extracted from various foods including seafoods can also be analysed. Sulfonamides are widely used as feed additives for fattening of animals. Combined with inhibitors of dihydrofolate reductase such as trimethoprim, tetromoprim, or pyrimethamine sulfonamides are also used in veterinary medicine for the treatment of intestinal infections, mastitis, pulmonitis and other diseases. Sulfonamide residues may therefore occur in food of animal origin such as milk and meat. The carcinogenic sulfamethazine represents a threat to human health. The recently published EC Regulation No. 675/92 established a maximum residue limit (MRL) of 100 ppb for sulfonamides in meat and a preliminary MRL of 100 ppb in milk.

For the detection of sulfamethazine physico-chemical methods such as HPLC are used because the microbial inhibition assays lack sensitivity and desired specificity. But due to high cost and instrumentation associated with HPLC procedures its use for routine screening has been limited.

Using RIDASCREEN Sulfamethazine test it is possible to detect as little as 10 ppb of this residue in samples. RIDASCREEN and RIDA are trade marks of R-Biopharm GmbH, Germany.

Principle:

The basis of the test is the antigen-antibody reaction carried out in an ELISA mirotiter plate. The microtiter wells are coated with sheep antibodies directed against anti-sulfamethazine rabbit IgG (i.e. antibodies to antibodies against the enzyme-sulfamethazine conjugate). Anti-sulfamethazine antibodies, sulfamethazine-enzyme conjugate, and the sulfamethazine standard or sample solution are added. Free sulfamethazine and sulfamethazine-enzyme conjugate compete for the anti-sulfamethazine antibody binding sites. At the same time , the anti-sulfamethazine antibodies are also bound by the immobilised sheep antibodies. Any unbound enzyme conjugate is then removed in a washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) are added to the wells and incubated. Bound enzyme conjugate converts the colourless chromogen into a blue product. The addition of the stop reagent changes the colour from blue to yellow. The colour intensity is measured at 450nm. The absorption is inversely proportional to the sulfamethazine concentration in the sample.

Materials:

Each kit contains sufficient materials for 96 measurements. Each kit contains:

(1) Microtiter plate, one.

(2) Sulfamethazine standard concentrates, 300μl each; 0, 10, 30, 90, 270, and 810 ppb.
(3) Conjugate. Peroxidase -sulfamethazine conjugate.
(4) Anti-sulfamethazine antibody concentrate.
(5) Substrate. 7ml solution.
(6) Chromogen. 7ml solution.
(7) Stop reagent. 14ml solution. Contains 1M sulphuric acid.
(8) Buffer 1 (20-fold concentrate).
(9) Buffer 2, concentrate, standard dilution buffer for sample.

Other material required but not provided with the kit:

Equipment: microtiter plate reader, centrifuge, rotary evaporator, stomacher, shaker, graduated pipettes, and micropipettes.

Preparation of Samples:

As per instructions provided with the kit.

Preparation of Working Solutions:

As per instructions provided with the kit.

Test Procedures:

(a) Add 50μl of diluted enzyme conjugate to the bottom of each well.
(b) Add 50μl of standard or prepare sample to the separate wells.
(c) Add 50μl of diluted antibody solution to each well. Mix well and incubate for 2 hours at room temperature.
(d) Pour out the contents of the wells by inverting over an absorbent paper.
(e) Fill all the wells with 250μl distilled water and pour out the liquid as in step 5. Repeat 2 more times.
(f) Add 50μl of substrate and 50μl of chromogen to each well. Mix and incubate min at room temperature in the dark.
(g) Add 100μl of stop solution to each well. Mix and measure the absorbance at nm against an air blank.

Results:

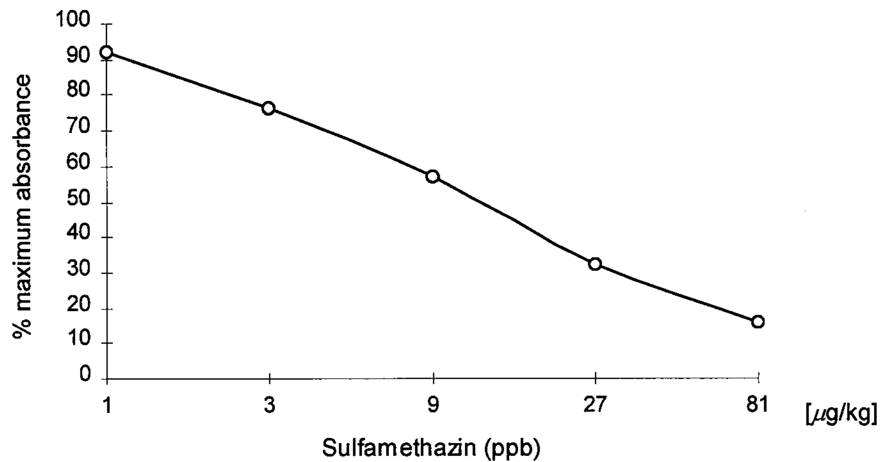
The mean values of the absorbance values obtained for the standard and the samples are divided by the absorbance value of the first standard (zero standard) and multiplied by 100. The zero standard is thus is made equal to 100% and the absorbance values are quoted in percentages.

The values calculated for the standard are plotted as shown in the following figure. The calibration curve should be virtually linear in the 1 - 27 μg/kg(ppb) range.

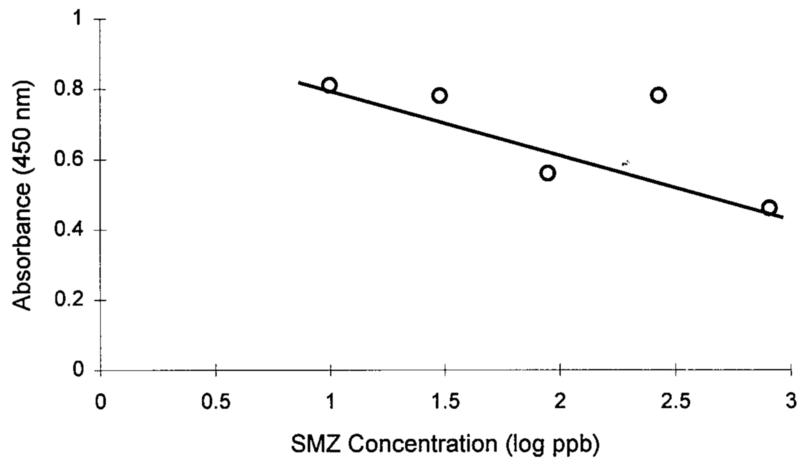
$$\frac{\text{Absorbance standard (or sample)}}{\text{Absorbance zero standard}} \times 100 = \%\ \text{Absorbance}$$

The sulfa methazine concentration in µg/kg corresponding to the absorbance each sample can be read from the calibration curve.

Calibration curve of RIDASCREEN Sulfamethazine kit



Test Results of RIDASCREEN Sulfamethaxine Kit



Std. SDZ (ppb)	SMZ (log ppb)	Absorbance (450 nm)
10	1.0	0.81
30	1.48	0.78
90	1.95	0.56
279	2.43	0.78
810	2.91	0.46